REDUCTIVE METABOLISM OF ALIPHATIC TERTIARY AMINE N-OXIDES

by

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Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification at this or any other university or institute of learning.

Pamela Tien (signed)

28-05-1999 (dated)

Abstract

This study is based on a proposal concerning the feasibility of using aliphatic tertiary amine *N*-oxides as antiarrhythmic agent prodrugs. Lignocaine was selected as a candidate for prodrug development, because the *N*-oxide is a non-active, polar derivative of lignocaine and the drug of choice for ventricular arrhythmia, a symptom associated with ischaemic episodes leading to regions of transiently hypoxic heart tissue. An HPLC analytical method was developed to study the metabolism of lignocaine *N*-oxide. The rapid and sensitive analysis of lignocaine and its metabolites was demonstrated with good reproducibility, stability and high recovery. In this study, it was identified that lignocaine *N*-oxide can be reduced to its active parent compound, lignocaine with no other metabolites detected in the absence of oxygen. Under anaerobic conditions, no further metabolism of lignocaine was demonstrated in rat liver microsomes and heart S9 fractions suggesting no secondary metabolites were formed. The reduction of lignocaine *N*-oxide has been shown to be both enzymic and non-enzymic, NADPH dependent, oxygen sensitive and can be suppressed by CO, CN^- and protein denaturation. Under anaerobic conditions, *in vitro* lignocaine *N*-oxide reduction was found to occur in NADPH supplemented rat liver homogenates, microsomal suspensions; rat heart homogenates, cytosolic solutions; human phenotyped cytochrome P450 isoforms; purified enzymes- cytochrome P450 reductase, xanthine oxidase, deoxymyoglobin and NADPH/ ascorbate reduced protohaem (haemin). This reaction can be suppressed through the chemically mediated decrease of P450 and b_5 levels in rat liver microsomes. Previous studies demonstrated that lignocaine *N*-oxide was non-active in aerobic rat heart *in vivo* and was potent under ischaemic conditions. In this study, high recovery of lignocaine *N*-oxide was found in the urine of normal rats suggesting low metabolism of the prodrug in oxic tissues. However, in hypoxic isolated rat hearts, lignocaine *N*-oxide was found to be reduced to lignocaine. The data presented suggested that the bioactivation of lignocaine *N*-oxide could be regulated by the prevailing oxygen tension in the ischaemic arrhythmic heart. Essentially the prodrug activation of lignocaine *N*-oxide may be triggered by the ischaemic state of the heart and terminated as the oxygen content in the heart returns to a more normal level. A controlled release and site-specific active drug delivery of lignocaine *N*-oxide, a hypoxia-mediated antiarrhythmic agent, may thus be achieved.

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Chapter 1 Introduction

1. 1 Hypoxia and oxygen mediated tissue damage

1.1. 1 Hypoxia induced tissue injury

Hypoxia is a common and serious aspect of many disease processes. It occurs in cells when there is a subnormal oxygen concentration. This may alter the cellular biochemical and physiological functions and produce pathological changes.

In clinical practice, hypoxia occurs when the arterial oxygen pressure falls below the normal range of 75 to 100 Torr.[1] Acute hypoxia can occur following an asthmatic attack, cardiac arrest, trauma, shock, stroke and carbon monoxide poisoning. Ischaemic damage due to acute hypoxia to the heart and brain results in high morbidity. Cardiovascular and pulmonary disorders, inflammation, chronic wounds and many other diseases which result in decreased blood flow and oxygen supply can produce chronic hypoxia which will cause damage to tissues and organs.

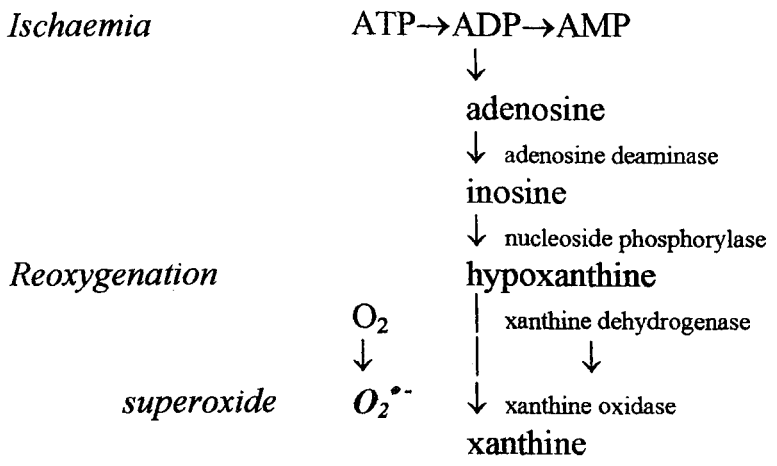
Hypoxia alters cellular metabolism and cell functions.[1,2] There is inhibition of both oxygen dependent enzymic reactions and mitochondrial oxidative phosphorylation. These metabolic changes can lead to cell membrane dysfunction and progressive development of irreversible cell injury. Toxic cell injury can be observed in the morphologic, metabolic and genetic events involved and on the effect of altered ion homeostasis. Apoptosis, autophagocytosis, acidosis, ion deregulation and cell necrosis may occur and led to irreversible tissue damages.

After hypoxia, reperfusion with oxygen can produce further tissue damage due to oxidative phosphorylation stopping, cellular ATP (adenosine triphosphate) levels dropping and increased concentrations of calcium and low molecular weight iron

complexes.[3] Reperfusion injury of ischaemic tissue induces the generation of free radicals and other toxic oxygen species. Reactive oxygen species such as superoxide anions ($O_2^{\bullet -}$) and hydrogen peroxide may be important mediators of cell injury in the post-ischaemia reperfusion *via* the oxidation of membranes or the alteration of critical enzyme systems leading to pathophysiological disorders.[4, 5] These reactive oxygen species play an important role in the pathology of cerebral ischaemia,[6] irradiation damage,[7] intestinal ischaemia,[8] inflammatory[9] and pulmonary disorders.[10]

Xanthine oxidase is reported to play an important role in ischaemia-reperfusion injury. Xanthine dehydrogenase can be converted into xanthine oxidase in ischaemic conditions and in the presence of the substrates hypoxanthine or xanthine. Xanthine oxidase will reduce molecular oxygen to $O_2^{\bullet -}$ and hydrogen peroxide that can be reduced further to the hydroxyl radical ($^{\bullet}OH$). During hypoxia, the process of hypoxanthine being converted to xanthine is triggered by the breakdown of ATP, ADP (adenosine diphosphate) to AMP (adenosine monophosphate), adenosine, inosine and finally to hypoxanthine.[6, 11] Upon reperfusion, xanthine oxidase and its requisite substrates (hypoxanthine and/or xanthine) will be presented in high concentrations and consequently result in the formation of reactive oxygen species.

The tissue damage can be inhibited by free radical scavengers, superoxide dismutase and catalase. The presence of xanthine oxidase inhibitors (*e.g.* allopurinol and its metabolite oxypurinol) can also decrease tissue damage indicating that xanthine oxidase generation of oxygen free radicals is a cause of ischaemic-reperfusion injury.[12, 13]



Scheme 1-1 Major pathways of adenosine nucleotide catabolism during myocardial ischaemia.

1.1. 2 Cardiac hypoxia and tissue injury

According to the World Health Organisation estimates in 1996, non-communicable diseases account for at least 40% of all deaths in developing countries and 75% in industrialised countries, where cardiovascular diseases are the first cause of mortality.[14] Myocardial arrhythmia among all cardiovascular diseases is the most frequent cause of the high morbidity and mortality. Although the immediate causes of such arrhythmias are often uncertain, a significant proportion have occurred after acute myocardial ischaemias/hypoxias or infarction and can lead to "sudden cardiac deaths", particularly ventricular fibrillation.[15]

Myocardial ischaemia occurs because of a reduction in the coronary blood flow which is so severe that insufficient oxygen is delivered to prevent respiration shifting from aerobic to anaerobic. When ischaemia is of sufficient severity and persists long enough, so that myocytes become irreversibly injured and undergo cellular necrosis, the myocardial infarction occurs. As seen in Figure 1-1, oxygen deficiency induces metabolic changes, including decreased ATP, decreased pH, and lactate accumulation in ischaemic myocytes. The altered metabolic milieu leads to impaired membrane transport with resultant rearrangements in intracellular electrolytes. An increase in cytosolic Ca^{2+} may trigger the activation of proteases and phospholipases with

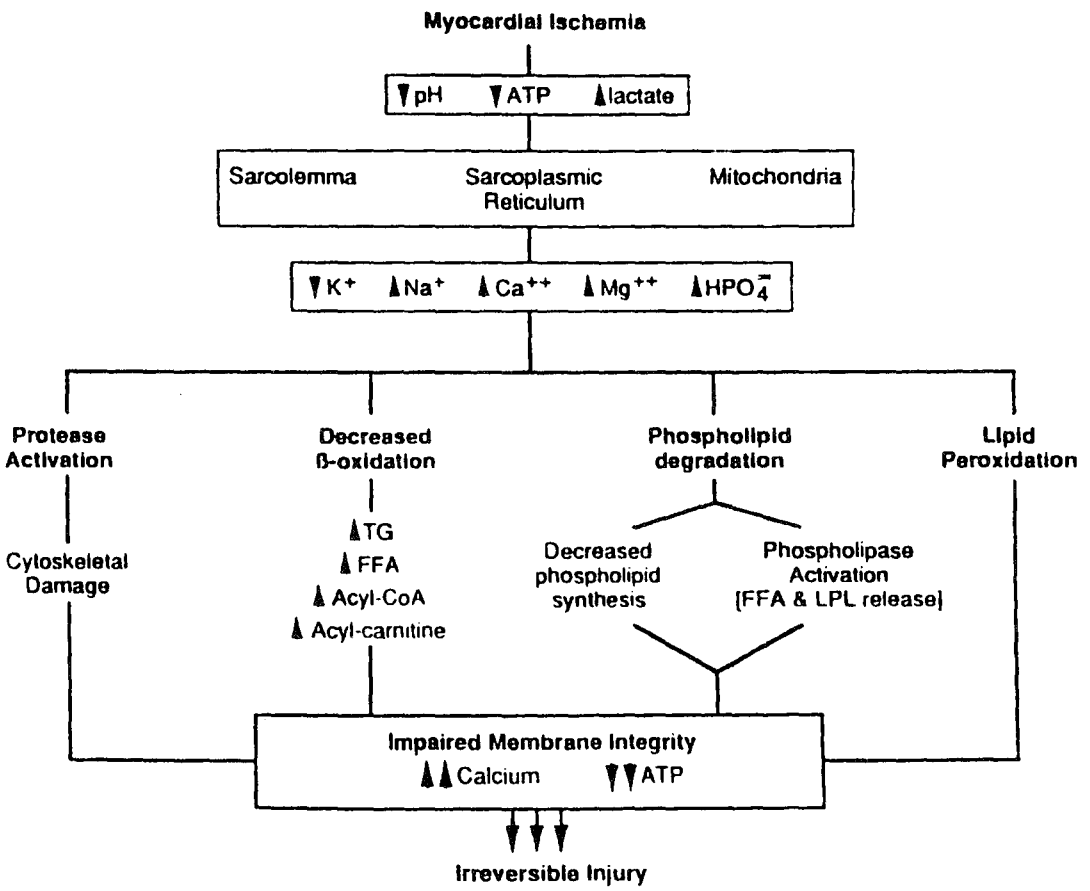


Figure1-1 Postulated sequence of alterations involved in the pathogenesis of irreversible myocardial ischemic injury.

From: Buja, LM Lipid abnormalities in myocardial cell injury. Trends Cardiovasc. Med. 1991, 1, 40-45.

resultant cytoskeletal damage and impaired membrane phospholipid imbalance. Lipid alterations include increased phospholipid (PL) degradation with release of free fatty acids (FFA) and lysophospholipids (LPL), and decreased phospholipid synthesis.

Lipid peroxidation occurs as a result of attack by free radicals produced at least in part by the generation of excess electrons (e^-) in oxygen-deprived mitochondria. Free radicals also may be derived from metabolism of arachidonic acid and catecholamines, metabolism of adenine nucleotides by xanthine oxidase in endothelium (species dependent), and activation of neutrophils and macrophages. The irreversible phase of injury appears to be mediated by severe membrane damage produced by phospholipid loss, lipid peroxidation, and cytoskeletal damage.

Reperfusion of a heart subjected to either global or regional ischaemia may also produce active oxygen species and create myocardial damage.[16] Conditions of ischaemia and reperfusion can be seen during open heart procedures in cardiac surgery (*e.g.* cardiopulmonary bypass) and heart transplantation. It has been demonstrated that xanthine dehydrogenase is converted to xanthine oxidase and can induce myocardial reperfusion injury.[17, 18]

1.1. 3 Clinical treatment of myocardial hypoxia/ischaemia

As cardiac arrhythmia is a major cause of death in patients with cardiac disease, it is evident that to control the incidence of sudden cardiac deaths, treatment should be directed primarily towards the prevention or suppression of the cardiac arrhythmia. Therefore, the administration of antiarrhythmic agents has become a major form of treatment in the management of cardiac arrhythmias.

Various methods of classifying anti-arrhythmic agents have been employed although the most widely used classification of anti-arrhythmic drugs is that proposed by Vaughan Williams[19] and later modified by Harrison.[20] This classification is based

largely on *in vitro* electrophysiological effects of the drugs on myocardial cells. The action potential involved in the contraction of cardiac muscle consists of several phases controlled by ionic movements across the myocardial cell membrane.

Class I agents include reagents which directly interfere with depolarisation of the cell membrane (membrane-stabilising agents) by blocking the fast inward current of sodium ions into cardiac cells; they also have local anaesthetic properties. They are subdivided into 3 further groups according to their effects on factors such as the duration of the cardiac action potential, the rate of change of the depolarisation phase of the cardiac action potential, the fibrillation threshold, conduction properties, and atrial and ventricular refractoriness. Class Ia drugs slow the rate of change of the depolarisation phase of the action potential, moderately prolong the repolarisation phase, and prolong the PR, QRS, and QT intervals on electrocardiogram (ECG) recordient. Typical agents of this class are disopyramide, procainamide and quinidine. Class Ib drugs have a limited effect on the rate of change of the depolarisation phase of the action potential, can shorten the repolarisation phase, shorten the QT interval, and elevate the fibrillation threshold. Typical agents of this class are lignocaine, mexiletine, phenytoin and tocainide. Class Ic drugs markedly slow the rate of change of the depolarisation phase of the action potential, have little effect on the repolarisation phase, and markedly prolong the PR and QRS intervals. Typical agents of this class are flecainide and propafenone. Class II drugs are the beta-adrenoceptor blocking agents. Class III drugs prolong the repolarisation phase of the action potential; amiodarone is a typical member of this class. Class IV drugs block the slow inward calcium current (calcium-channel blockers) although not all drugs that fall into the broad general category of calcium-channel blockers share the same specific properties; verapamil is a typical agent of this class. This classification system for anti-arrhythmic agents is summarised in Table 1-1.

The precise diagnosis of the type of cardiac arrhythmia is a prerequisite for proper management.[21] The choice of therapeutic treatment is often varied due to different type of arrhythmias and the concern of potential toxicity or precautions from individual differences. This may be attributed to the narrow therapeutic index of the

Table 1-1 Classification and activity of antiarrhythmic agents

Activity of Anti-arrhythmic Agents				
	Effect on rate of change of depolarisation phase of cardiac action potential	Effect on repolarisation phase of cardiac action potential	Beta-blocking activity	Calcium-channel blocking activity
Class Ia disopyramide procainamide quinidine	Slow	Moderately prolong	–	–
Class Ib lignocaine mexiletine phenytoin tocainide	Limited effect	Shorten	–	–
Class Ic flecainide propafenone	Markedly slow	Little effect	–	–
Class II beta blockers	–	–	+	–
Class III amiodarone	–	Prolong	–	–
Class IV verapamil	–	–	–	+

From : Reynolds, J. E. Martindale, The Extra Pharmacopeia, 30th ed., pp57, The Pharmaceutical Press, London, 1993.

"-" no effect.
"+" positive effect.

antiarrhythmic agents and the different pharmacokinetic/pharmacodymic properties of each individual (age, sex, disease, diet and other factors). In some instances, measurements such as pacing or direct current shock may be employed in addition to pharmacotherapy. The choices for antiarrhythmic agents in treating supraventricular arrhythmia and ventricular arrhythmia are listed in Table 1-2. Several types of drugs are effective in supraventricular arrhythmia. The main drugs used are amiodarone, disopyramide, procainamide, quinidine, verapamil and beta blockers (*e.g.* propanolol, atenolol). In ventricular tachyarrhythmias, including sustained ventricular tachycardia and ventricular arrhythmias following myocardial infarctions that warrant drug administration, lignocaine administered intravenously is the agent of choice.[22]

Adverse side effects are commonly found to accompany the antiarrhythmia treatment.[23] There is, overwhelming evidence to suggest that many antiarrhythmic drugs can and do sometimes provoke or aggravate arrhythmias and do kill patients.[24, 25, 26] In view of this, there is a need for new effective antiarrhythmic agents as most of the presently available drugs have limited efficacy, adverse side effects, inconvenient dosing regimes and interactions with other drugs.

Furthermore, the main aim of clinical treatment of preventing recurrent myocardial ischaemic attack is not only to prevent the myocardial ischaemias but also to prolong the survival, free of stroke and myocardial infarction that leads to high mortality rates. Anticoagulant (*e.g.* heparin) and antiplatelet (*e.g.* aspirin, dipyridamole) drugs are used to reduce the risk of stroke. Surgical approaches (*e.g.* carotid endarterectomy and extracranial-to-intracranial bypass procedures) have been used to improve blood flow and oxygen supply to both heart and brain. Therefore, the quest for a safer and more effective antiarrhythmic agent is extremely important.

Table 1-2 Choice of antiarrhythmic agents in the treatment of cardiac arrhythmias.

Choice of Anti-arrhythmic Agent			
	Acute termination	Alternatives for acute termination	Long-term suppression
Supraventricular Arrhythmia	Verapamil	Amiodarone Beta Blocker Disopyramide Procainamide Quinidine	Amiodarone Beta Blocker Disopyramide Procainamide Quinidine Verapamil
Atrial flutter and fibrillation	Digoxin (to slow ventricular response)	Beta Blocker or Verapamil (as additional or alternative therapy)	Beta Blocker Digoxin Disopyramide Procainamide Quinidine Verapamil
Atrioventricular re-entrant tachycardia (paroxysmal supraventricular tachycardia)	Adenosine	Beta Blocker or Verapamil	Beta Blocker Disopyramide Quinidine Verapamil
Ventricular Arrhythmia	Lignocaine (Phenytoin or Lignocaine if digoxin-induced)	Amiodarone Beta Blocker Bretylium Disopyramide Flecainide Mexiletine Procainamide Quinidine	Amiodarone Beta Blocker Disopyramide Flecainide Mexiletine Procainamide Quinidine

From: Reynolds, J. E. Martindale, The Extra Pharmacopeia, 30ed., pp.57, The Pharmaceutical Press, London, 1993

1.1. 4 Lignocaine

Lignocaine, a local anaesthetic, is a class Ib agent and is used to control serious ventricular arrhythmias. It is the drug of choice for the treatment of ventricular premature beats, especially after acute myocardial infraction and is used parenterally for the acute treatment of arrhythmias that arises from cardiac surgery and catheterisation. It is also useful in the prevention of recurrence of ventricular fibrillation and to treat ventricular tachycardia. Lignocaine is used in treating digitalis-induced arrhythmias and heart failure, and has proved to be useful in the correction of those arrhythmias that persist despite administration of potassium and withdrawal of cardiac glycosides.[27, 28]

Lignocaine administration is limited to the parenteral route due to its rapid first pass metabolism in the liver. The most widely used regime for the administration of lignocaine consists of an intravenous bolus injection of 100 mg followed by a continuous infusion at 1-2 mg/min but this does not maintain plasma concentration in the therapeutic range.[29, 30] Therapeutic plasma concentration of lignocaine can be rapidly achieved and maintained by following the bolus injection with a constant infusion. Although it is highly effective and is extensively used in the clinic, lignocaine possesses dose-related side effects including dizziness, nausea, drowsiness, speech disturbances, beside numbness, muscle twitching, confusion, vertigo and/or tinnitus. Also, serious neurotoxicities such as sinus arrest, severe bradycardia or complete arterioventricular (AV) block may occur following lignocaine treatment.[31, 32]

Despite the above side effects, lignocaine has been the cornerstone of acute treatment for ventricular arrhythmias for nearly 20 years and there is yet no comparable drug for long term administration to treat established arrhythmias or prevent the development of arrhythmias. It has been demonstrated that lignocaine exerts its myocardial protective effect by scavenging highly cytotoxic reactive species of oxygen generated during ischaemia and reperfusion. This membrane stabilising property of lignocaine

can be attributed partially to its removal of hydroxyl radical and singlet oxygen that are implicated in membrane lipid peroxidation.[30]

The metabolic fate of lignocaine has been studied extensively in experimental animals and humans.[33, 34, 35, 36] In rat hepatic microsomes, lignocaine is sequentially *N*-deethylated to give first monoethylglycinexylidide (MEGX), and then glycine xylidide (GX). Both compounds were shown to possess antiarrhythmic activity and neurotoxicity. [37, 38, 39, 40] Lignocaine is also metabolised *via* hydroxylation of the aromatic ring to give 3-or 4-hydroxylignocaine (3-OH-LIG or 4-OH-LIG), respectively.[41, 42, 43] These reactions are catalysed by P450s in hepatic microsomes. Lignocaine and nine of its metabolites: MEGX, GX, 3-OH-LID, 4-OH-LIG, 3-OH-MEGX, 4-OH-MEGX, 4-OH-GX, xylidine (XY) and 4-OH-XY, except lignocaine *N*-oxide, have been detected in human plasma and urine.[44]

1. 2 Prodrugs

1.2. 1 Prodrug concept

A prodrug is generally defined as a chemical which is pharmacodynamically inert and of low toxicity but which under physiological conditions may undergo spontaneous rearrangement or degradation or be converted enzymatically to a pharmacologically active drug. The prodrug concept has been recognised since the 1950's[45] and has since been reviewed extensively.[46, 47, 48, 49, 50]

1.2. 2 Prodrug approach

A molecule with optimal structural configuration and physicochemical properties for achieving the desired therapeutic response at its target site does not necessarily possess the best molecular form and properties for its delivery to its point of ultimate action. A basic requisite for the prodrug approach to be useful in solving drug delivery problems is the ready availability of chemical moieties that satisfy the prodrug requirements. The most prominent of these is the reconversion of the prodrug to the parent drug *in vivo*. This prodrug-drug conversion may take place before absorption (*i.e.* in the gastrointestinal tract), during absorption, after absorption or at the specific site of drug action in the body, depending upon the specific purpose for which the prodrug is designed. Ideally, a prodrug should be converted into its parent compound in a controlled manner in order to achieve the highest efficacy and the lowest toxicity. Approaches to prodrug development have involved enhancement of bioavailability and passage through various biological barriers, and increased site-specificity.

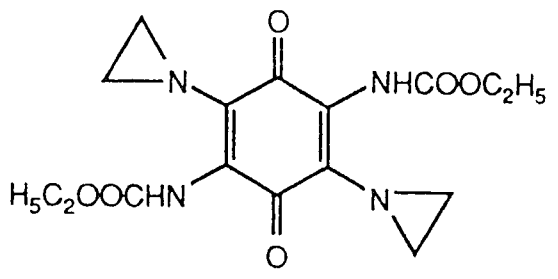
The conversion or activation of prodrugs to the parent drug molecules in the body can take place by a variety of reactions. The most common prodrugs are those requiring a hydrolytic cleavage mediated by enzymatic catalysis. Active drug species containing hydroxyl or carboxyl groups can often be converted into prodrugs from which the active forms are regenerated by esterases within the body. In other cases, active drug substances are regenerated from their prodrugs by biochemical reductive or oxidative processes. The application of bioreduction to the development of prodrugs of anticancer agents is described in the next section (1.2.3).

1.2. 3 Prodrug application: bioreductive anti-cancer agents

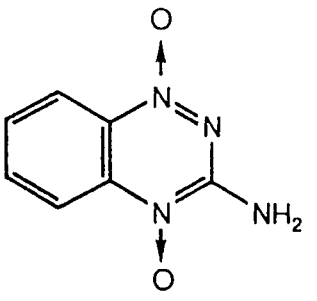
Anticancer prodrugs have been synthesised to have more favourable physicochemical properties which may (a) increase the stability (b) optimise the route of

administration, (c) alter the distribution, (d) bypass drug resistance, (e) improve pharmacokinetics and (f) achieve selective activation in target cells. The approach of targeting bioreducing anticancer agents that are activated selectively by hypoxic tumour cells have been widely studied. [51, 52, 53, 54] In chemotherapy, bioreductive prodrugs are activated by metabolic reduction in the hypoxic tumour cells to form highly effective cytotoxins. Tumour selectivity exploits the presence of hypoxia in tumours since oxygen can reverse the activating step of the bioreduction thereby greatly reducing the activity of the drug in most normal tissues. Molecular oxygen in cells acts principally as a terminal electron acceptor in oxidative phosphorylation and as a source of dioxygen for processes such as steroidogenesis and haem degradation. Flavoprotein based dehydrogenases and oxidoreductases mediate the flow of electrons from reducing equivalents in the form of the nicotinamide dinucleotide coenzymes, NADH and NADPH, to dioxygen and other endogenous acceptor molecules. Various chemotherapeutic agents are substrates for certain flavoprotein enzymes that ordinarily participate in intermediary metabolism. The selectivity depends on the level of expression of these particular enzymes in tumour cells for which the drug can act as a substrate. These include DT-diaphorase,[55] various P450 isozymes,[56, 57] cytochrome P450 reductase[58] and xanthine oxidase.[59, 60] Also, the enzymic expression in cells can be modulated by internal factors such as oxygen deficiency, intracellular pH changes, and even by the malignant phenotype itself.

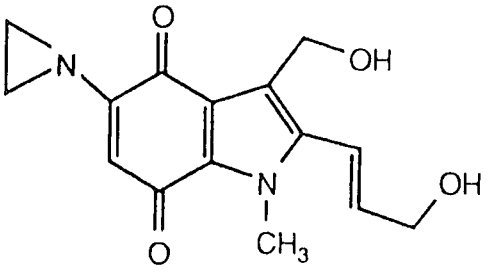
The bioreductive anticancer agents which have been extensively investigated fall into several subgroups predominantly the quinones, nitro compounds and *N*-oxides.[61, 62] The chemical structures of some bioreductive anti-tumour prodrugs are shown in Figure 1-2. Benzotriazene di-*N*-oxide (tirapazamine, SR4233)[63] and the nitro compounds (RSU 1069 and RB 6145)[64] are capable of killing hypoxic cells. The bioreductive mechanisms of quinones (Diaziquone (AZQ), E09 and mitomycin C)[65, 66] relies on bioreductive activity of alkylating functional groups that crosslink DNA. However, there are major problems with these types of cytotoxic agents. First, they are likely to be dependent on chronic hypoxia to maintain activity since diffusion of the reactive species or parent compound away from the hypoxic tissue, or tissue



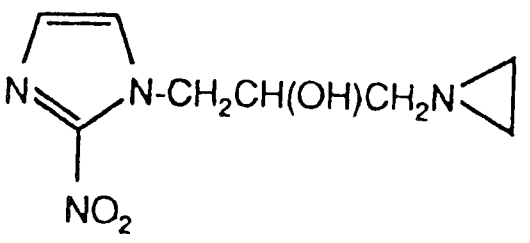
(a) diaziquone (AZQ)



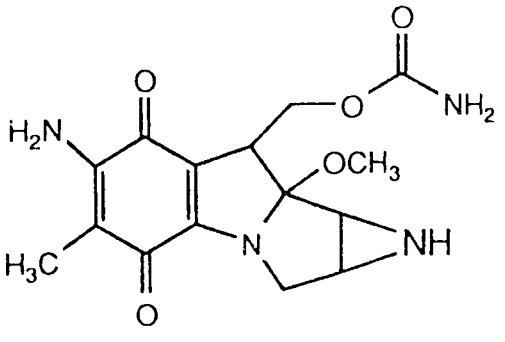
(d) SR4233



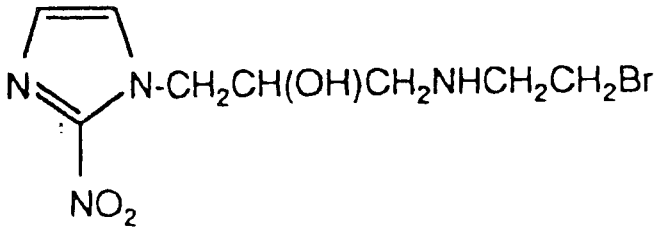
(b) E09



(e) RSU 1069



(c) mitomycin C

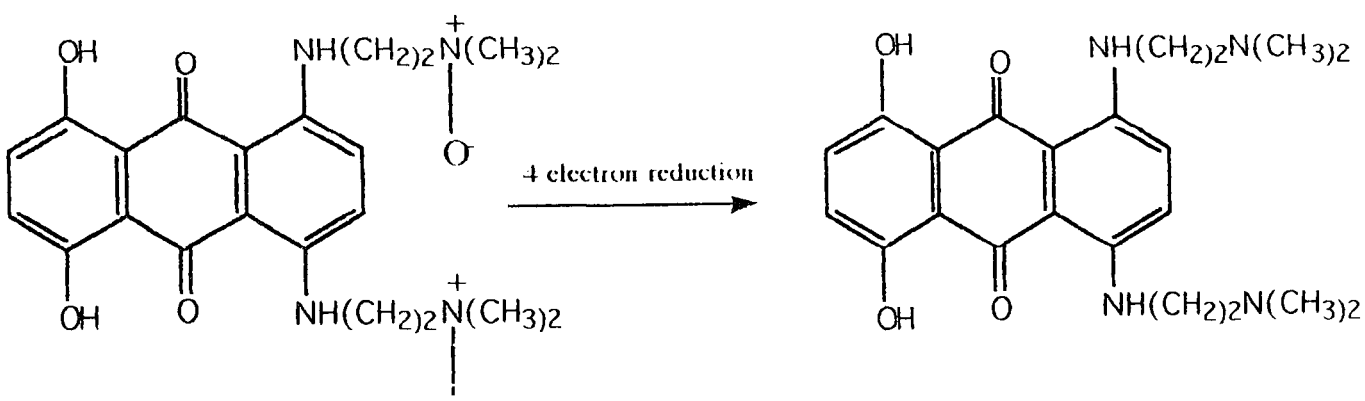


(f) RB 6145

Figure 1-2 The chemical structures of some bioreductive anti-tumor prodrugs: (a) diaziquone (AZQ), (b) E09, (c) mitomycin C, (d) benzotriazene di-*N*-oxide (tirapazamine, SR4233), (e) RSU 1069 and (f) RB 6145.

reoxygenation will result in inactivity. Secondly, the intracellular target for most bioreductive agents is considered to be DNA and yet none of these agents accumulate in the nucleus since they do not possess specific, non-covalent DNA binding affinity. Also, although acute hypoxia in tumours could be a useful aspect of tumour physiology with regard to drug targeting, it would be advantageous if a bioreducibly activated agent could be developed that maintains activity even after oxygen reperfusion takes place.

Aliphatic amine *N*-oxides with bioreductive potential and affinity of DNA binding have been developed as a new class of cytotoxic prodrugs. This concept is illustrated using the alkylaminoanthraquinones that are a group of cytotoxic agents with DNA binding affinity dependent on the cationic nature of these compounds.[67] The actions of the alkylaminoanthraquinones involve drug intercalation into and inhibition of topoisomerase, an enzymes crucial to the processing of DNA prior to cell division. A di-*N*-oxide analogue of mitoxantrone, 1,4-bis([2-(dimethylamino-*N*-oxide) ethyl]amino) 5,8-dihydroxy anthracene-9,10-dione (AQ4N) has been shown to possess no intrinsic binding affinity for DNA, low toxicity and can be anaerobically reduced *in vitro* to its parent compound, 1,4-bis([2-(dimethylamino)-ethyl]amino)-5,8-dihydroxyanthracene-9,10-dione (AQ4), with up to 1000 fold increase in cytotoxic potency (Figure 1-3). AQ4 is stable under oxic conditions. Studies indicate that the *in vivo* antitumour activity of AQ4N is manifest under conditions that promote transient hypoxia and/or diminish the oxic tumour fraction. The advantage of utilising the reductive environment of hypoxic tumours to reduce *N*-oxides is that unlike nitro or quinone based compounds, the resulting products will remain active even if the hypoxia that led to bioactivation is transient or the active compounds, once formed, diffuse away from the hypoxic tumour regions. Furthermore, the DNA affinic nature of the active compounds should ensure their localisation in tumour tissue.[68] The concept of using aliphatic amine *N*-oxides as hypoxic selective bioreductive prodrugs has been adapted in this project to study the hypoxic/ischaemic bioreduction of an *N*-oxide of an antiarrhythmic tertiary amine as a potential prodrug.



AQ4N AQ4

Figure 1-3 The bioreduction of 1,4-bis([2-(dimethylamino-*N*-oxide) ethyl] amino)5,8-dihydroxyanthracene -9,10- dione (AQ4N) to its parent compound, 1,4-bis([2-(dimethylamino)-ethyl]amino)5,8-dihydroxyanthracene-9,10-dione (AQ4).

1. 3 Aliphatic tertiary amine *N*-oxides

N-Oxides were discovered at the end of the last century. They are polar products of the two-electron oxidation of tertiary amines. Early this century the natural occurrence and, later on, the biological functions and interactions of *N*-oxides became known. The chemistry, pharmacology, biochemistry, toxicity; and the possible use of amine *N*-oxides as therapeutic agents have been reviewed.[68, 69] Historical reviews of *N*-oxide formation by biological systems oxides have also been written.[70, 71]

Many naturally occurring and synthetic *N*-oxides as well as *N*-oxide metabolites of amines have been reported to possess pharmacological activity[72] or toxicity. Example of *N*-oxide which posses biological activity include (i) alkaloids, *e.g.* the *N*-oxides of strychnine, atropine, hyoscyamine, scopolamine and morphine (ii) chemotherapeutics, *e.g.* *N*-oxides of benzotriazines,[73] and quinoxalines;[74] (iii) antibiotics, *e.g.* iodinine[75] and aspergillic acid;[76] (iv) psychotropic drugs, *e.g.* chlordiazepoxide, active *N*-oxide metabolites of chlorpromazine and imipramine; (v) methemoglobin forming compounds, *e.g.* the *N*-oxide of *N, N*-dimethylaniline;[77] and (vi) carcinogenic agents, *e.g* 4-nitroquinoline *N*-oxide,[78] and indicine *N*-oxide, a pyrrolizidine alkaloid isolated from *Heliotropium indicum*, characterised as an antitumour agent and had been studied extensively.[79] These amine *N*-oxides are either active themselves or can be reduced to their active parent compounds and may be categorised as "prodrugs".

There are essentially four types of *N*-oxides: (a) tertiary aliphatic amine *N*-oxides, (b) *N,N*-dialkylarylamine *N*-oxides, (c) heteroaromatic *N*-oxides and (d) imino *N*-oxides (or nitrones). All these four classes of *N*-oxides (Figure 1-4) can be reduced to the corresponding tertiary amines in a variety of *in vitro* and *in vivo* studies. [80, 81, 82, 83]

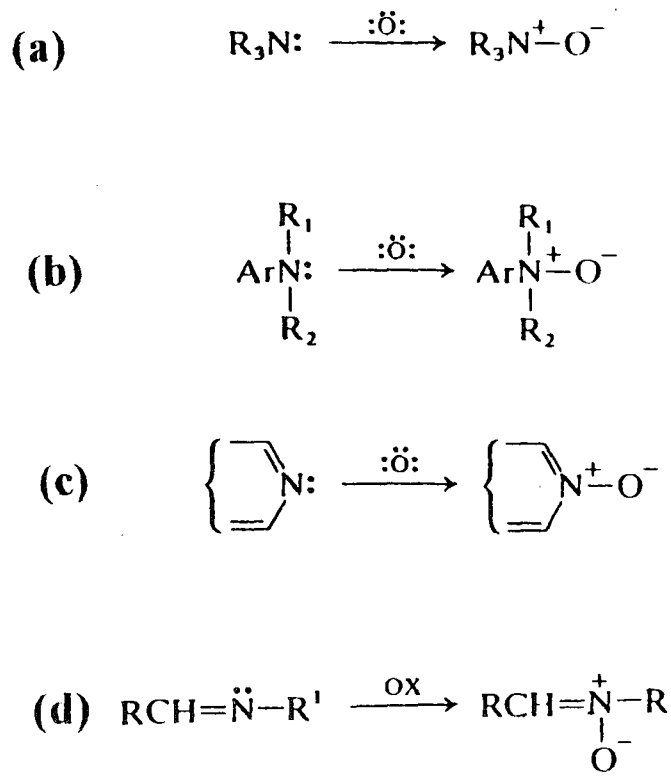


Figure 1-4 *N*-oxygenation of different types of tertiary amines to *N*-oxides.
(a) tertiary amine *N*-oxide
(b) N, N-dialkylarylamine *N*-oxide
(c) Heteroaromatic *N*-oxide
(d) Imino *N*-oxides (nitrones)

1.3. 1 The formation of *N*-oxides: *N*-Oxidation

Nitrogen-containing substances are the most common of all of organic compounds of pharmacological interest. Pharmacological or therapeutic interest in nitrogen-containing compounds can be dated back to the application and toxicity studies of alkaloids and their extracts such as stimulants (cocaine, nicotine, caffeine), analgesic/ euphoriants (morphine), tranquilizers (reserpine) and decongestants (ephedrine). In spite of the extensive information on their pharmacology, the major pathways of metabolism were not thoroughly investigated until the mid-1950s.

N-oxygenation is a common metabolism route of tertiary amines. The formation of *N*-oxides through *N*-oxygenation of tertiary amines involves the nitrogen lone pair of electrons. The metabolism of organic nitrogen compounds by *N*-oxidation was first described by Lintzel[84] who reported that trimethylamine administered to humans was largely excreted as trimethylamine *N*-oxide. Subsequent studies with more complex amines showed that *N*-oxidation is a general route for metabolism of many nitrogen containing compounds; metabolites produced by an oxidative attack on a nitrogen centre are frequently detected both *in vivo* and *in vitro*.[85, 71] Interest in this pathway was further stimulated by the pioneering studies of the Millers [86] and Kiese[87] on the role of *N*-oxidation in the metabolic activation of arylamides to more toxic and carcinogenic derivatives.[88] The chemistry and enzymology of oxidation of nitrogen compounds have been extensively reviewed.[89, 90, 91, 92]

Nitrogen compounds may undergo *N*-oxygenation that includes *N*-hydroxylation and *N*-oxidation to form *N*-hydroxy compounds and amine *N*-oxides,[93, 94] respectively.

N-Oxides may be formed as metabolites of drugs and other foreign compounds containing a tertiary aliphatic, tertiary alicyclic, *N,N*-dialkylaryl, or heteroaromatic amino functionality.[92 , 95, 96, 97]

Metabolic *N*-oxidation of tertiary amines has been generally considered to be catalysed by both flavin-containing monooxygenase[98, 99] and the cytochrome P450 system.[93] Tertiary amines are *N*-dealkylated or *N*-oxygenated and the nature of the products formed is dependent on the structure of the amine as well as on the catalytic mechanism of the monooxygenases involved.[100] Lindeke and Cho proposed an *N*-oxidation followed by rearrangement to α-C-hydroxylation product as a possible mechanism for *N*-dealkylation.[91] However, an *N*-oxide product has been demonstrated to be a major metabolite but not an obligatory intermediate in the dealkylation of tertiary amine.[101] Gorrod proposed a theory relating to the multiplicity of *N*-oxygenation enzymes and the pKa of nitrogen containing substrates.[95, 102] Less basic amines (less nucleophilic) were suggested to be better substrates for the cytochrome P450 dependent monooxygenase, whilst more basic amines were better substrates for the flavin containing monooxygenase. Exceptions exist but this hypothesis still serves as a basic principle in the determination of enzyme mediated amine oxidation pathways.

1.3. 2 Metabolism of tertiary amine *N*-oxides

This study will concentrate on the reduction of tertiary aliphatic amine *N*-oxides. The early systematic work on *N*-oxide reduction was from Bickel,[85,103] who summarised the complexities of this metabolic reaction. Essentially, *N*-oxide reduction has been reported to be enzymatic, non enzymatic or both; aerobic or anaerobic; heat-labile or heat-stable; dependent on, or not connected with xanthine oxidase; or dependent on various co-factors.

Non-enzymic reduction of *N*-oxide has also been reported. Trimethylamine *N*-oxide can be reduced by Fe^{2+}, cysteine, or reduced glutathione.[104] Similarly chlorpromazine *N*-oxide was reduced by Fe^{2+}.[105] Haem iron also had an effect on *N*-oxide reduction. The *N*-oxides of *N,N*-dimethylaniline, *N,N*-dimethylaminoazo-benzene and

imipramine are readily reduced in erythrocytes, or in oxygenated solutions of haemoglobin.[101,106] This haem-iron mediated *N*-oxide reduction may play a role in *N*-oxide metabolism *in vivo*.

The studies by Dajani *et al* [107] on nicotine-1'-*N*-oxide indicated the complex nature of the *N*-oxide reduction pathway. *N*-Oxide reduction was observed in both the soluble and the microsomal fraction of rat liver and small intestine, the reductase being non-specific with regard to the requirement for NADH or NADPH. From various induction, inhibition and heat inactivation studies, Dajani concluded that nicotine-1'-*N*-oxide reduction was mediated not only by cytochrome P450, but also linked partially to NADPH-dependent flavoprotein enzymes, such as NADPH-cytochrome P450 reductase or xanthine oxidase.

The work done by Kato and co-workers[108, 109, 110] was the first that resulted in the clarification of the enzymology of aliphatic amine *N*-oxide reduction. Tertiary amine *N*-oxides were demonstrated to be reduced back to tertiary amines by rat liver subcellular preparations in the presence of NADPH or NADH. Most of the reductase activity was located in the liver microsomal fraction. The microsomal NADPH dependent *N*-oxide reductase activity was inducible by phenobarbitone pre-treatment, oxygen sensitive, and inhibited by carbon monoxide, n-octylamine, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) and various other P450 inhibitors, suggesting the involvement of cytochrome P450 in *N*-oxide reduction. Sugiura *et al.* [108] studied the combination of NADPH and NADH on *N*-oxide reduction which prompted a closer investigation of the NADH-dependent *N*-oxide reductase activity. Iwasaki *et al.*[110] have provided proof of the ability of cytochrome P450 to mediate *N*-oxide reduction. Studies with purified P450 have shown that both P450 and P448 will reduce tiaramide *N*-oxide at comparable rates, and that P450 isolated from rat, rabbit and *Pseudomonas putida* had comparable reducing activity despite widely different substrate specificities in oxidation reactions.

Cytochrome P450 involvement has been further demonstrated in the case of indicine *N*-oxide reduction with rat liver microsomes by Powis.[66] Indicine *N*-oxide is a pyrrolizidine alkaloid *N*-oxide with anticancer activity. Tiaramide *N*-oxide and indicine *N*-oxide reduction by rat hepatic microsomal fractions occurs under anaerobic conditions and is completely inhibited by carbon monoxide. However, in contrast to tiaramide *N*-oxide reduction, NADH is almost as effective as NADPH in supporting indicine *N*-oxide reduction. The NADH-dependent reduction is inhibited by potassium cyanide and by acetone, whereas the NADPH-dependent reduction is inhibited only slightly (3%) by potassium cyanide and stimulated by acetone. Phenobarbitone pre-treatment produces a selective increase in the maximal rate of the NADPH-dependent *N*-oxide reduction. Powis [66] rationalised these observations in terms of a different isoenzymic form of cytochrome P450 catalysing indicine *N*-oxide reduction, this isoenzyme being able to accept electrons from NADH to affect *N*-oxide reduction, but not contributing to oxidative microsomal drug metabolism.

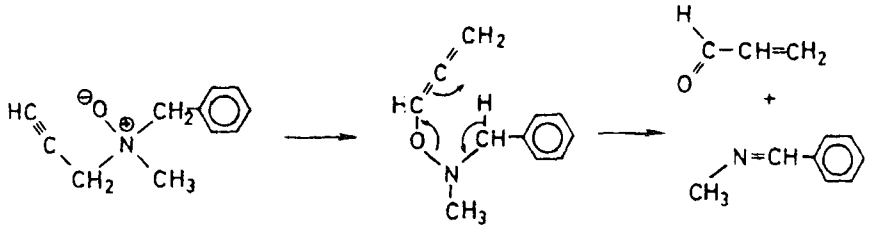
In addition to the microsomal reduction of *N*-oxide, the cytosol also appears to be a site for reduction of certain tertiary aliphatic amine *N*-oxides. A purified rat liver xanthine oxidase preparation was identified as being capable of the xanthine dependent reduction of benzylamine *N*-oxide.[111] The enzymic reduction seems to involve xanthine oxidase and/ or cytochrome P450 in crude rat liver preparations. The *N*-oxide reduction was blocked by allopurinol, an inhibitor of xanthine oxidase, and n-octylamine, an inhibitor of cytochrome P450 *N*-oxide reductase. Johnson and Ziegler identified an NADPH-dependent cytosolic *N*-oxide reductase for *N,N*-dimethylaminoazo-benzene reduction.[112] This protein was shown to be a cytosolic, NADPH-dependent *N*-oxide reductase. Although P450 and enzyme involvement in *N*-oxide reduction have been well recognised, their substrate specificities, mechanisms and their relative contributions to *N*-oxide reductions *in vivo* are still not fully known.

The *in vivo* metabolism of *N*-oxides has not been studied extensively or systematically. Early studies on trimethylamine *N*-oxide suggested that *N*-oxide reduction of this compound is not a major route of metabolism.[84] The *in vivo*

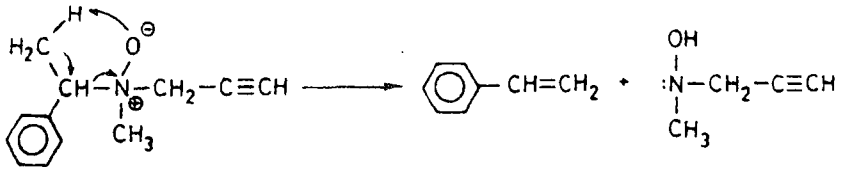
metabolism of chlorpromazine *N*-oxide, amitriptyline *N*-oxide, indicine *N*-oxide and imipramine *N*-oxide have been reported to go through *N*-dealkylation, hydroxylation as well as *N*-oxide reduction.[113, 114, 115, 116] Ziegler proposed that tertiary amines would undergo rapid *N*-oxidation in oxygenated tissues.[117] Thus, the concurrent occurrence of *N*-oxide reduction and *N*-oxidation makes the investigation of *in vivo* *N*-oxide reduction to its parent tertiary amine difficult. The reversible *N*-oxidation of these tertiary amines and their further oxidation/ hydroxylation may be accounted for the absence of *N*-oxide reduction pathway *in vivo*. Although *N*-oxides may undergo *in vivo* reduction, a reversible oxidation/reduction may also occur and thus *N*-oxides may have long half-lives.

N-oxides have been reported to undergo various non-enzymic rearrangements, *i.e.* Cope and Meisenheimer rearrangements.[118] In the Cope rearrangement, tertiary amine *N*-oxides with β hydrogen atoms to the nitrogen decompose to secondary hydroxylamines and an alkene. The Meisenheimer rearrangement involves the migration of a group from nitrogen to oxygen in tertiary amine oxides having no β hydrogen atoms. It has been reported that a tertiary amine *N*-oxide, pargyline *N*-oxide, undergoes both (a) Meisenheimer and (b) Cope rearrangements.[119]

(a)



(b)



These rearrangements may occur during the isolation of the metabolites. A similar rearrangement has been reported from studies on the metabolism of lignocaine. A cyclic metabolite, N^1-ethyl-2-methyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone, attributed to reaction between monoethylglycinexylidide (MEGX) and ethanol has been identified.[120]

1.3. 3 Involvement of cytochrome p450 in tertiary amine *N*-oxide reduction

In biological systems, compounds can undergo metabolism through oxidation or reduction of the nitrogen derivatives to form polar metabolites that are readily eliminated in the urine. The oxidation and reduction reactions can be catalyzed by the cytochrome P450 systems. The involvement of cytochrome P450 in metabolising tertiary amine *N*-oxide reduction is investigated in this study.

1.3.3. 1 The cytochrome P450 enzymes

Cytochrome P450 is a b-type haemoprotein that contains iron protoporphyrin IX as a prosthetic group, and exhibits an unusual absorption maximum at 450 nm for its ferrous carbon monoxide complex.[121,122] Cytochrome P450 can be found in plants, animals and microorganisms, and takes part in a great variety of biochemical oxidation/reduction reactions. Cytochrome P450 exists as multiple forms.[123, 124, 125] Some including mitochondrial based steroid-hydroxylating and the bacterial cytochrome P450s are characterised by strict substrate specificity. Other isoforms are located in the liver microsomal fractions that possess a wide substrate specificity due to the existence of multiple cytochrome P450 isozymes, and hence can oxygenate non-polar substances of different chemical natures. By converting a hydrophobic compound into a more polar product, cytochrome P450 facilitates the elimination of non-polar molecules from the body. At least two proteins, NADPH-specific flavoprotein and cytochrome P450 haemoprotein, are necessary for the catalytic activity in the presence of NADPH as an electron donor.[126, 127, 128, 129] The flavoprotein reductase, contains both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) prosthetic groups and functions in the transfer of reducing equivalents from NADPH to the cytochrome.

The various cytochrome P450 isoforms have been classified by Nebert.[130, 131, 132, 133] The characteristic functions and properties of multiple forms of cytochrome P450 in

human and animals have been extensively studied and reviewed by Guengerich.[134, 135, 136, 137, 138, 139] Individual differences in the isoforms of cytochrome P450 may be due to various factors such as race, sex, age, diet, disease, life style,[140] as well as exposure to a variety of inducing or suppressing agents including drugs, chemicals, and carcinogens.[141, 142,143, 144, 145]

To date, more than 150 different cytochrome P450 forms have been identified. The recommended nomenclature system unambiguously identifies each form as a unique protein, based on the percentage homology of primary amino acid sequences. Proteins in the same cytochrome P450 family are at least 40% homologous and are greater than 55% in the cytochrome P450 subfamilies. When describing the cytochrome P450 gene, *"CYP"* is italicised and designates the gene for cytochrome P450. The first Arabic numeral designates the cytochrome P450 family. This is followed by a capital letter designating the subfamily, and another Arabic numeral to distinguish members within a subfamily. Cytochrome P450 isoform mediated enzymic reactions have been studied in many pathways of metabolism[146, 147] *e.g.* *CYP*1A2 and *CYP*3A in imipramine *N*-oxygenation and C-hydroxylation[148] and *N*-hydroxylation of dapsone by cytochrome *CYP*3A4.[149]

1.3.3. 2 Mechanism of cytochrome P450 mediated oxidation and reduction

The overall monooxygenase reaction catalysed by this system is:

$$RH + NAD(P)H + O_2 + H^+ \rightarrow ROH + NAD(P)^+ + H_2O$$

The mechanisms of the catalytic reactions of the cytochrome P450 enzymes have been extensively studied and characterised into several chemical steps (Figure 1-5a).[150] The process begins with the substrate binding to the ferric form of the cytochrome P450 enzyme. ①Oxidised cytochrome P450 [Fe(III)] can react with a molecule of substrate (R) to form the enzyme-substrate complex. ② The P450-

subtrate complex then undergoes a one electron reduction catalysed by NADPH dependent cytochrome P450 reductase. ③ The resultant complex of the reduced (ferrous) haemoprotein and substrate [Fe(II)-R] can react with oxygen to form a ternary complex called oxycytochrome P450 (R-P450-O_2). ④ A second electron than enters the complex. This electron can come from either NADPH dependent cytochrome P450 reductase or NADH cytochrome b_5 reductase. Following ⑤ the protonation of the [Fe(II)-O_2^+] complex and the release of one molecule of water, ⑥ the oxygenated [Fe(V)=O] or [Fe(III)-O] is then converted to [Fe(III)] and the formation of an oxidised substrate. ⑦The series of cytochrome P450-directed reactions is concluded when the oxidised substrate dissociates from cytochrome P450, regenerating the ferric haemoprotein. The sequence of reactions described above has most of the common features for all types of cytochrome P450, regardless of source.

Although cytochrome P450 is known predominantly as a mixed function oxidase, there is some evidence in the literature[110] that this haemoprotein can act as a reductase in the absence of oxygen. A two electron reaction mechanism was proposed earlier by Kato and Sugiura for the cytochrome P450 mediated tertiary amine *N*-oxide reduction steps (Figure 1-5 b) including imipramine *N*-oxide, tiaramide *N*-oxide and *N,N*-dimethylaniline *N*-oxide. [108] The reaction starts with a one electron reduction of cytochrome P450 *via* NADPH-cytochrome P450 reductase. The *N*-oxide substrate is directly co-ordinated to the reduced cytochrome haem. Followed by a further one electron reduction of the reduced cytochrome P450-*N*-oxide (substrate) complex, the tertiary amine *N*-oxide is reduced to give the corresponding tertiary amine and one molecule of water.

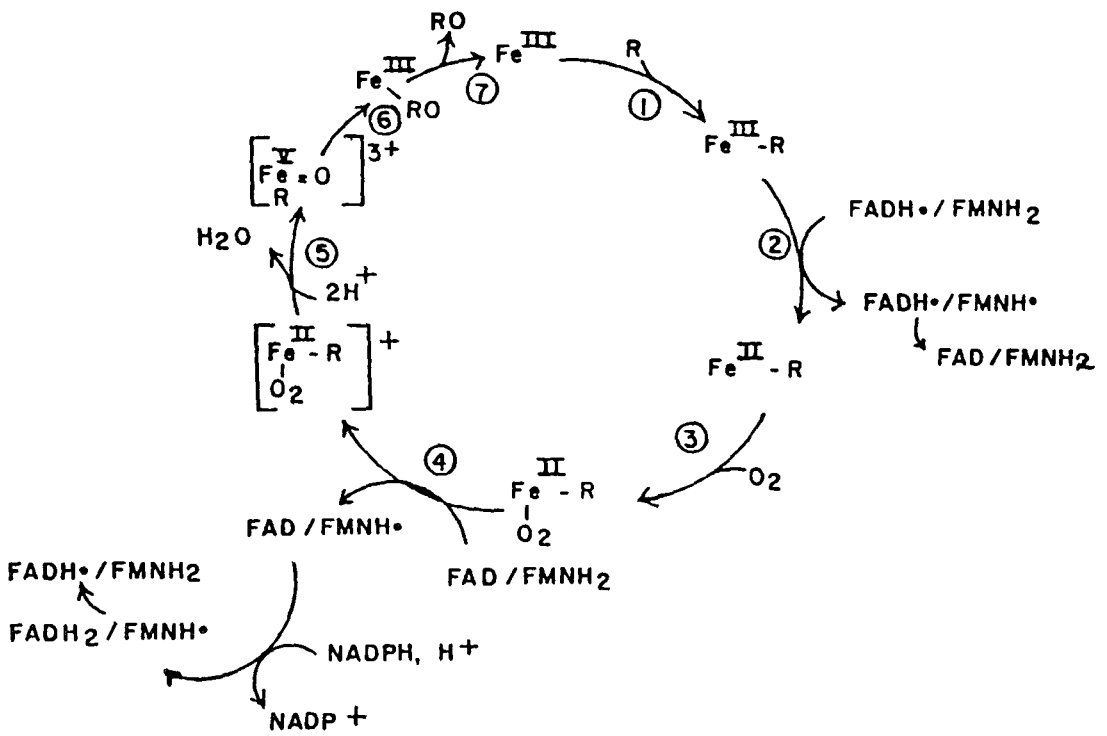


Figure 1-5 (a) Proposed catalytic cycle for reduction and oxidation of cytochrome P450. (Guengerich and Maconald, 1984)

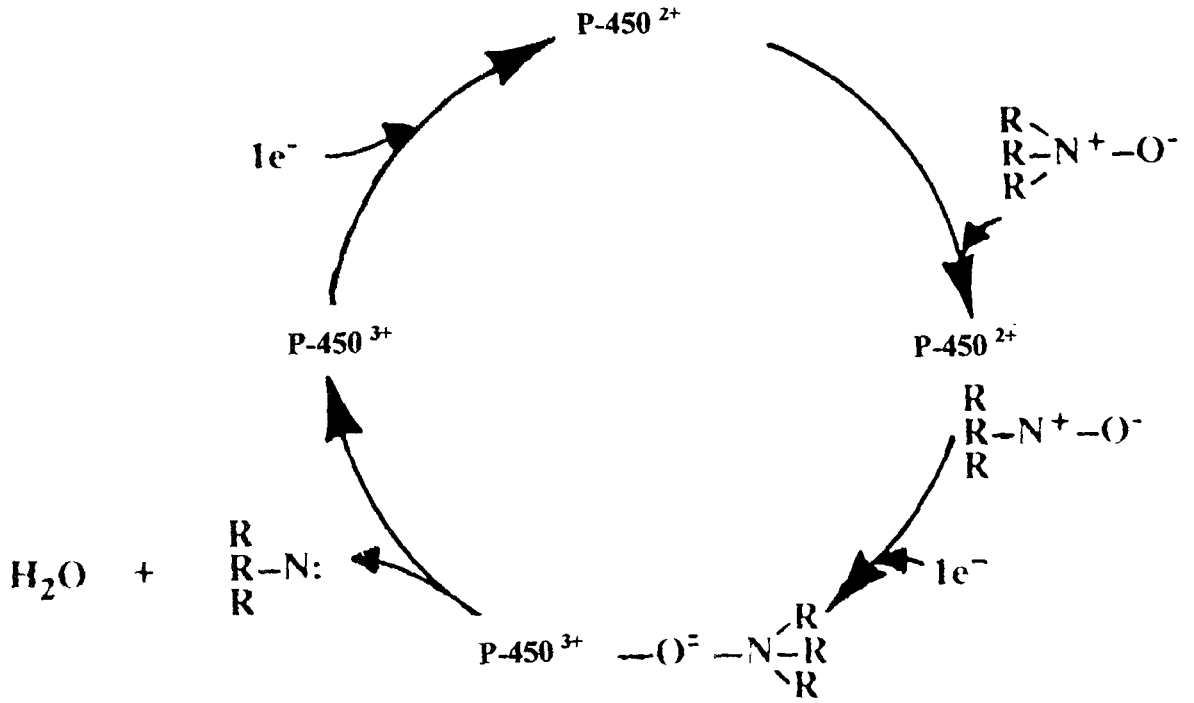


Figure 1-5 (b) Proposed catalytic cycle for the reduction of tertiary amine N-oxides by liver microsomal cytochrome P450. (Sugiura, Iwasaki and Kato, 1976)

1.3.3. 3 Type I and Type II binding of substrate to cytochrome P450

In the presence of a cytochrome P450 enzyme system, certain electron-rich nitrogenous substrates, such as *N*-oxides, will form spectrally defined complexes. The substrate binding to cytochrome P450 induces some conformational changes around the haem centre and increases the redox potential of the haem iron.[151, 152] This binding often perturbs the P450 spectrum in the visible region causing a characteristic substrate-binding spectrum. A pronounced spectral characteristic of all porphyrin derivatives is that upon excitation they give rise to an intense $\pi \sim \pi^*$ transition at about 400 nm (the Soret band), which is due to delocalization extending throughout the tetrapyrrole ring system, and some other characteristic longer wavelength bands. The spectral characteristics are sensitive to changes in the haemporphyrin as well as changes in its environment. Thus, changes in the haem iron oxidation states or spin (ligation) states of the iron atom can be distinguished as the conformational changes in the protein surrounding the haem group.[153] This inherent chromophoric property has made spectrophotometric methods a useful tool in analysing cytochrome P450 and other haemoproteins including myoglobin and peroxidases.[154, 155, 156]

The most significant property of the haemoporphyrins is their capacity to bind molecular oxygen. This is the basis of oxygen transportation by myoglobin and haemoglobin as well as the activation of molecular oxygen by various cytochromes including cytochrome P450. Oxygen binding can occur only when the iron of the haem is in its reduced [Fe(II)] state. In cytochrome P450 mediated reactions, reduction of the haem iron of the enzyme-substrate complex generates a high-spin (pentacoordinated) ferrous form which is capable of binding oxygen. Upon the binding of oxygen, cytochrome P450 forms a low-spin (hexacoordinated) ferrous enzyme-substrate oxygen complex (Figure 1-6). The substrate-P450 complexes are termed the "metabolic intermediary complex" (MI complexes).[157]

In studies of cytochrome P450-mediated reactions, there are two main types of spectral perturbations in the Soret region. These changes are classified as type I and

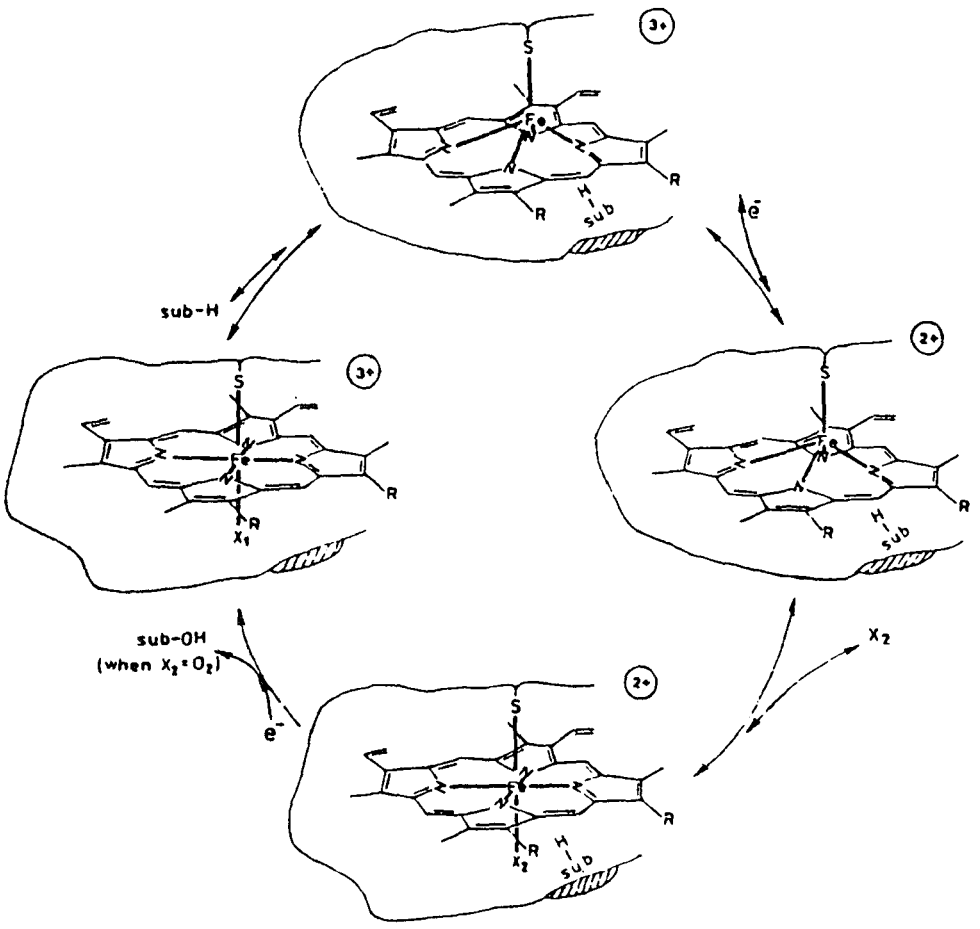


Figure 1-6 Schemic reaction cycle of cytochrome P450, showing proposed ligand and spin state changes. (Lindeke, B., Paulsen-Sorman, 1988).

type II spectral changes. Spectrally these two types of perturbations are essentially mirror images of each other and both result from the interaction of the enzyme in its oxidised (Fe^{3+}) form with various substrates (Figure 1-7). Substrates have been classified as type I or type II depending on their ability to cause either type I or type II spectral changes upon addition to the enzyme. Type I spectral changes have been considered to be associated with metabolism, *i.e.* to be the optical manifestation of the binding of substrate to the protein moiety of the enzyme; while type II spectral perturbations are believed to reflect a direct interaction between the substrate and the haem iron.

In the binding of nitrogenous compounds to ferric cytochrome P450, the type II spectral change, as characterised by an absorption peak at 425-435 nm and a trough at 390-405 nm, has been shown to arise from ligand binding of nitrogen, in which *sp2* or *sp3* non bonded electrons are sterically accessible to the haem iron of cytochrome P450. The type I spectral change, as documented by the appearance of an absorption peak at 385-390 nm and an absorption minimum at 420 nm, is believed to arise from the displacement of the sixth haem ligand in the cytochrome P450 molecule through substrate binding to a hydrophobic domain in the apoprotein.[158, 159, 160, 161]

1.3.3. 4 The effect of cobalt on haem oxygenase and cytochrome P450

Haem oxygenase is a microsomal enzyme which catalyses the NADPH-cytochrome P450 reductase dependent degradation of haem to biliverdin at the expense of three oxygen molecules.[162, 163] It is a monomeric protein that has a molecular weight in the range of 30 to 35kDa and exists as two isoenzymes- HO-1 and HO-2.[164] Early studies demonstrated that several metals, such as cobalt, tin, and cadmium could act as haem oxygenase inducers.[165, 166] Administration of cobalt was shown to increase the

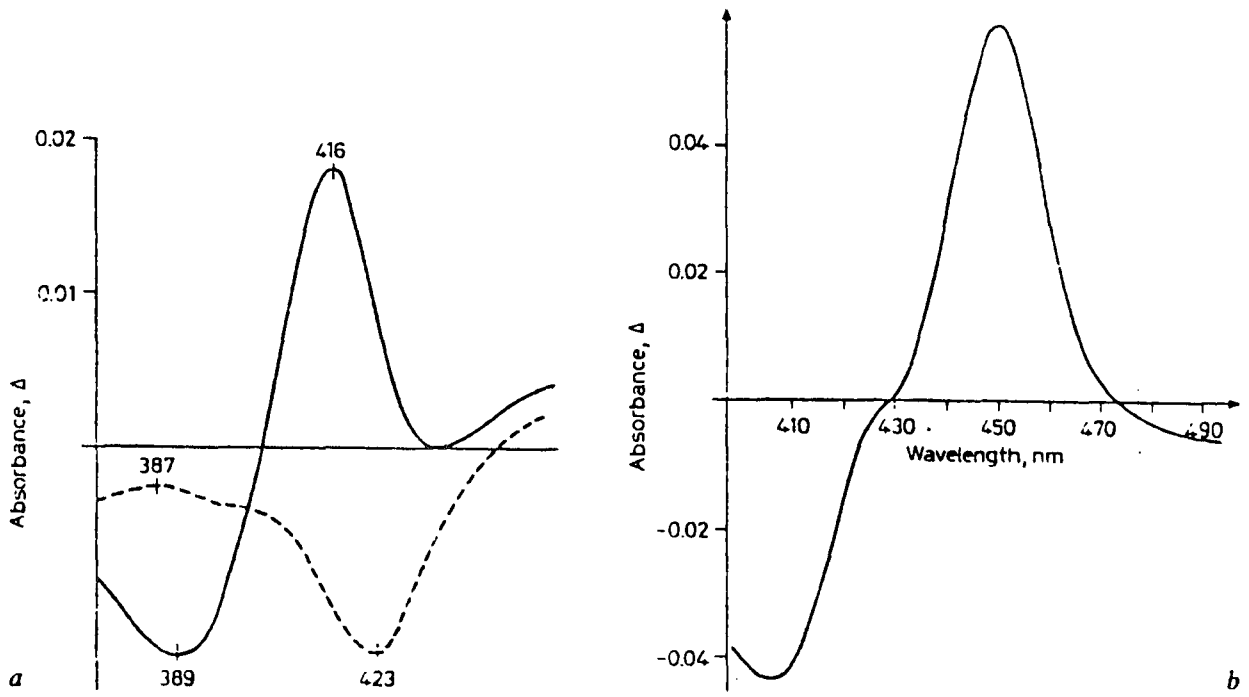


Figure 1-7 (a) Aerobic difference spectra of liver microsomes with the iron in its oxidized (Fe^{3+}) state, in the presence of N-hydroxyamphetamine (—= type II) and N-hydroxy-phentermine (---= type I). (b) Difference spectra of liver microsomes with the iron in its reduced (Fe^{2+}) state, in the presence of carbon monoxide. (Lindeke, B., Paulsen-Sorman, 1988).

enzyme activity of haem oxygenase with a decrease in the microsomal content of cytochrome P450.[167] The characteristic, function, and isoforms of haem oxygenase has been extensively studied and reviewed by Maines.[168, 169]

Haem oxygenase has been identified as being decreased under various cellular stresses and disease states and is classified as an oxidative stress sensitive protein.[170] In hypoxic cells, the increase in haem oxygenase activity provides protection for cells against oxidative stress. This situation is observed in tumour hypoxic conditions and heart and kidney ischaemias.[171, 172] Oxidative stress has been reported to induce haem oxygenase in animal and human tissues; such induction can be regarded as an antioxidant defence mechanism.[173] Under stress conditions, haem oxygenase can decrease the levels of the haem and haemoproteins such as cytochrome P450 and protoporphyrinogen oxidase while increasing the tissue concentrations of bile pigments which possess antioxidant properties.[174] It may be valuable to apply cobalt treatment in studying the role of haem oxygenase and cytochrome P450 in *N*-oxide reduction. The ability of haem oxygenase to act as a reductase in hypoxic conditions that reduces *N*-oxides is considered and will be investigated in this project.

1. 4 Aliphatic tertiary amine *N*-oxides as antiarrhythmic prodrugs

1.4. 1 Lignocaine *N*-oxide

Lignocaine has been shown to reduce ventricular arrhythmias associated with myocardial infarction and ischaemic myocardial injury. The protective effect of lignocaine has been attributed to its membrane stabilising properties. Lignocaine is

the drug of choice in the acute treatment of myocardial arrhythmia. The high first-pass effect and narrow therapeutic index of this agent do, however limit its use. Ideally as a prodrug, lignocaine *N*-oxide, is expected to be pharmacologically inactive and relatively stable *in vivo* until it is reduced back to the parent compound, lignocaine, at the site of action (hypoxia selective). Lignocaine *N*-oxide, like most aliphatic amine *N*-oxides, is more polar and less potent (non-toxic) compared to its parent compound, lignocaine.[175] It would be suitable to use it as a putative hypoxia-selective antiarrhythmic prodrug to study the feasibility of this approach to drug targeting in the hypoxic/ischaemic heart.

Lignocaine, which is an aliphatic tertiary amine, could be expected to be metabolised through *N*-oxidation with the formation of a tertiary amine *N*-oxide. However, lignocaine *N*-oxide was identified, only as a minor metabolite of lignoaine, in rat liver microsomes.[176] In rat liver microsomes, lignocaine is *N*-deethylated to two active metabolites, monoethylglycinexylidide (MEGX) and glycine xylidide (GX); and can be hydroxylated to 3-or 4-hydroxylignocaine (3-OH-LIG or 4-OH-LIG).[37, 38, 39, 40, 44] The metabolic pathway of lignocaine is shown in Figure 1-8.

1.4. 2 Analysis of tertiary amine *N*-oxides

Aliphatic tertiary amine *N*-oxides are extremely water soluble, thermolabile, and unstable at extremes of pH. [72, 177] Despite this lability and the fact that the polarity of *N*-oxides makes their isolation from biological fluids difficult. Analytical techniques have been developed for the isolation, identification and quantification of the intact aliphatic tertiary amine *N*-oxides from biological media. The physical-chemical properties of the *N*-oxides influence the choice of assay method and probably account for the fact that direct analysis of tertiary amine *N*-oxides has not been studied in detail until recent years.

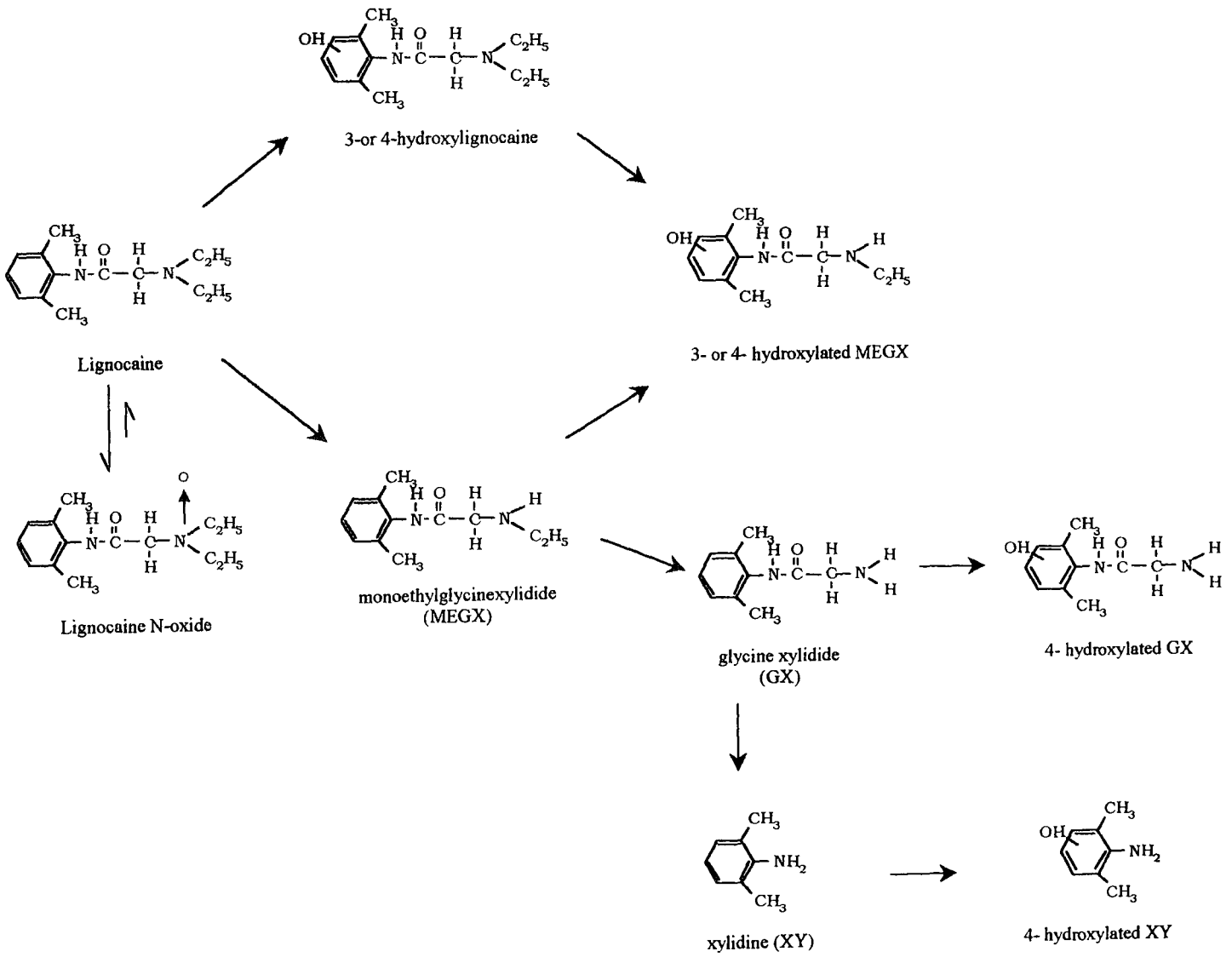


Figure 1-8 Scheme for lignocaine metabolism

Gas chromatography (GC) is a rapid and sensitive analytical method. Aliphatic tertiary amine *N*-oxides are highly polar and possibly thermally labile that need to be chemically modified before being analysed by GC. Unfortunately, aliphatic tertiary amine *N*-oxides have been shown to decompose during the chemical modification using acid anhydrides or acid chlorides to tertiary amines. Therefore, gas chromatography (GC), is not considered suitable for use in the aliphatic tertiary amine *N*-oxide metabolism studies.

High performance liquid chromatography (HPLC) has been successfully used in the direct identification and quantification of tertiary amine *N*-oxides.[178] Two important features of HPLC that facilitate the extraction, recovery and stability of *N*-oxides and metabolites in biological matrices are its operation at ambient environment and the capability of polar compound analysis. HPLC analysis of many *N*-oxides including diltiazem *N*-oxide,[179] several antihistamine *N*-oxides (brompheniramine *N*-oxide, chlorpheniramine *N*-oxide, pheniramine *N*-oxide and pyrilamine *N*-oxide),[180, 181] imipramine *N*-oxide,[182] chlorpromazine *N*-oxide[113,183] have been developed.

Analysis of lignocaine and its metabolites using HPLC [35], GC [184, 185] and gas chromatography/mass spectrometry (GC/MS) [186] is well documented. The separation and identification of lignocaine and lignocaine *N*-oxide has been reported by Prof. Patterson *et al.* In 1986 using thin-layer chromatography (TLC). However, no reports showing the quantification of lignocaine *N*-oxide by any analytical methods have been published. Thus, a priority to study lignocaine *N*-oxide as an antiarrhythmic prodrug was to develop a rapid and sensitive analytical method.

1. 5 The aims/objectives

The aim of this study is to investigate the fate of lignocaine *N*-oxide in biological systems as part of a feasibility study into its use as a bioreductive antiarrhythmic prodrug that functions selectively under hypoxic conditions to generate the active parent drug, lignocaine. The metabolism and enzymology of lignocaine *N*-oxide in heart and liver tissues will be carried out to assess the capability of the site specific biotransformation for this proposed antiarrhythmic prodrug. Preliminary studies *in vivo* will also be conducted to assess the systemic stability of lignocaine *N*-oxide. The objectives of this thesis are:

1. To develop and validate an HPLC analytical method for the determination of lignocaine *N*-oxide and its principal metabolites.

2. To determine aerobic and anaerobic metabolism of lignocaine *N*-oxide in rat liver and heart subcellular fractions and purified enzymes.

3. To study the mechanism of lignocaine *N*-oxide bioreduction with particular reference to haemoproteins including cytochrome P450, haem oxygenase and myoglobin.

4. To undertake preliminary studies *in vivo* concerning the metabolic fate of lignocaine *N*-oxide in rat isolated perfused heart.

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Chapter 2 Experimental

2.1 Materials

2.1.1 Materials

Lignocaine *N*-oxide, amiodarone *N*-oxide, diltiazem *N*-oxide and disopyramide *N*-oxide were synthesised under contract by Dr. D. Rathbone, Aston Molecules, Birmingham, U.K.. The compounds were shown to be authentic and pure by NMR, mass spectroscopy and elemental analysis.

Monoethylglycinexylidide (MEGX) was synthesised using the method of Keenaghan *et al.*, 1972 [36]. The purity and authenticity of this compound was demonstrated by IR spectroscopy and HPLC.

The following chemical and biochemical materials were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.): ammonium sulphate, calcium chloride ($CaCl_2 \cdot 6H_2O$), cobalt chloride ($CoCl_2 \cdot 6H_2O$), DEAE cellulose, disodium hydrogen phosphate, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), dimethylsulphoxide (DMSO), disopyramide, ferric chloride ($FeCl_3$), ethylenediaminetetraacetic acid (EDTA), ferrous sulphate ($FeSO_4 \cdot 7H_2O$), D-glucose, D-glucose 6-phosphate dehydrogenase, D-glucose-6-phosphate monosodium salt, haemin, 1-heptane sulphonic acid sodium salt, hydrogen peroxide, L-ascorbic acid, lignocaine hydrochloride, magnesium chloride ($MgCl_2 \cdot 6H_2O$), methylamine (4.0 M, aqueous solution), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate (NADPH), potassium chloride, potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium dithionite ($Na_2S_2O_4$), sodium chloride, sodium hydrogen carbonate ($NaHCO_3$), catalase, superoxide dismutase (SOD, xanthine oxidase and tris(hydroxymethyl)aminomethane.

Acetonitrile and methanol were of HPLC grade. Triethanolamine, acetic acid and hydrochloric acid (10 M) were analytical grade.

Celite (acid-washed) and Sephadex G-15 and G-100 were from Pharmacia (Herts, U.K.).

Purified rat liver Cytochrome P450 reductase was provided by Professor C. R. Wolf, Biomedical Research Centre, Ninewells Hospital Medical School, Dundee. Phenotyped human liver microsomes and analysis of data were provided by Dr. Colin Henderson, Imperial Cancer Research Foundation, Molecular Pharmacology Unit, Biomedical Research Centre, Level 5, Ninewells Hospital & Medical School, Dundee.

Human recombinant haem oxygenase (HO-1) was provided by Professor P. Ortiz DeMontellano, Department of Pharmaceutical Science School of Pharmacy, University of California at San Francisco, U.S.A..

2.1.2 Buffers and reagents

Tyrode's solution

Sodium chloride (40 g), D-glucose (10 g), magnesium chloride (1.016 g), sodium dihydrogen phosphate (0.25 g), sodium hydrogen carbonate (5 g), potassium chloride (1.864 g) and calcium chloride (1 g) were dissolved in 5 litres of distilled water to give a final pH of 7.4.

0.01 M Phosphate buffer (pH 7.4)

Appropriate volumes of 0.01 M disodium hydrogen phosphate (1.420 g in 1 litre of water) and 0.01 M potassium dihydrogen phosphate solutions (1.3609 g in 1 litre of water) were mixed to make a 0.01 M phosphate buffer at a final pH of 7.4.

0.1 M Tris-HCl buffer (pH 8.5)

Tris(hydroxymethyl)aminomethane (2.4 g) and sodium chloride (2 g) were dissolved in about 100 ml of water, the pH adjusted to 8.5 with 1 M sodium hydroxide or 1.0 M hydrochloric acid and the solution diluted with water to 200 ml.

NADPH generating system

The NADPH generating system consisted of NADP (10 μmole), glucose-6-phosphate (5 μmole), glucose-6-phosphate dehydrogenase (1 unit) and 1 μl magnesium chloride solution (50% w/v) in 100 μl of 0.01 M phosphate buffer (pH 7.4).

2.1.3 Preparation of subcellular fractions from rat liver and heart

Freshly prepared livers and hearts were obtained from adult male rats (Sprague-Dawley strain) provided by the Pharmacology section, De Montfort University. Homogenisation and ultracentrifugation (Beckman L8 ultracentrifuge) were used to prepare the subcellular fractions as follows.

Rat livers or hearts were excised and rinsed with ice-cold 0.01 M phosphate buffer (pH 7.4) to remove any blood. After the tissue was blotted dry, it was weighed, cut into small pieces and added to 0.01 M phosphate buffer (pH 7.4) of twice the volume of the wet organ weight. Suspensions containing finely sliced tissues were homogenised with a motor driven Teflon pestle in a Potter- Elvenjehm homogeniser by seven to ten repeated passes of the pestle at a speed of 800-1000 r.p.m.. The homogenate was then centrifuged at 4°C, with a fixed angle rotor head (8 x 50 ml), for 30 min at 10,000 g. The centrifuge tube after this stage contained a supernatant, and a pellet that consisted of nuclei, mitochondria, unbroken liver cells and red blood cells. The supernatant (S9) was retained and distributed into cellulose nitrate tubes

(13.5 ml) and further centrifuged at 140,000 g for 60 min, using a fixed angle (type 65) rotor head. Supernatant containing soluble cytosolic fraction and a pellet (microsomes from the liver and sarcosomes from heart) were obtained and stored in liquid nitrogen before use. The microsomal/ sarcosomal suspension was prepared by resuspending the pellet in a volume of 0.01 M phosphate buffer (pH 7.4) equal to the original liver/heart wet weight using a hand held Potter-Elvenjehm homogeniser. The protein content of each subcellular fraction was determined using the Bio-Rad protein assay (see Appendix I).

2.1.4 Purification of bovine myoglobin

Myoglobin of high purity was obtained as deoxymyoglobin from fresh bovine heart muscle and was shown to be free of metmyoglobin as an intermediate. All procedures were performed at about 4 °C as follows[188, 189, 190, 191].

Fresh bovine heart muscle (1.7 kg), free of connective tissue was homogenised with approximately 1 litre of 5 mM Tris-HCl buffer, pH 8.5 and the homogenate was centrifuged at 4000 g for 10 min. Appropriate amounts of 1 M Tris-HCl buffer or 1 M NaOH solution were added to the supernatant to adjust the buffer concentration to 20 mM, pH 8.5. Solid ammonium sulphate (ultrapure) was then added to achieve 70% saturation. After one hour, the precipitate, which was collected by centrifugation at 20,000 g for 15 min, was discarded. Addition of more ammonium sulphate to saturation gave a precipitate that was collected on Celite (acid-washed). The aqueous extract obtained by treatment of the Celite crude deoxymyoglobin mixture with 5 mM Tris-HCl buffer (pH 8.5), was applied to a column, equilibrated with 5 mM Tris-HCl buffer (pH 8.5), consisting of a top layer (6.5 x 5 cm) of Sephadex G-15 and a bottom layer (6.5 x 65 cm) of Sephadex G-100. The deoxymyoglobin was eluted from the column with the same buffer and dialysed overnight against the buffer. The deoxymyoglobin solution was then placed on a

column (6 x 10 cm) of DEAE cellulose equilibrated with 5 mM Tris-HCl buffer (pH 8.5), and washed with 1 column volume of the same buffer. Most of the deoxymyoglobin was seen to remain on the lower portion of the column and was removed by elution with 30 mM Tris-HCl buffer (pH 8.5). Following dialysis for 5 hours against 5 mM Tris-HCl buffer (pH 8.5) the deoxymyoglobin solution was placed on a column (6 x 1.5 cm) of DEAE cellulose (DE52) equilibrated in 5 mM Tris-HCl buffer (pH 8.5). Metmyoglobin was collected from the DE52 column in 5mM Tris-HCl buffer, pH 8.0. Deoxymyoglobin was further eluted from the column with 0.1 M Tris-HCl buffer, pH 8.5. Any metmyoglobin remaining was removed by repeating this procedure. Deoxymyoglobin was purified freshly before use.

Ferryl myoglobin was prepared from metmyoglobin using a 1.5 molar excess of H_2O_2 in 10 mM Tris-HCl buffer (pH 8.5).[192] Ferryl myoglobin was freshly prepared and quantified spectrophotometrically before use. All myoglobins were stored at -5 °C and protected from light. The concentration of myoglobin was measured by the Bio-Rad method as described in Appendix I.

2.2 Analytical methods

A series of reversed-phased HPLC columns with various packing materials (C6, C8, and C18) and different column length (15 cm and 25 cm) were tested. The constituents (*e.g.* phosphoric acid, acetic acid, citric acid, phosphate buffers, acetate buffers and triethanolamine) and pH value of the mobile phase were investigated to achieve good separation of lignocaine *N*-oxide and its metabolites. Different proportions of methanol and acetonitrile were added into aqueous solutions to acquire a satisfactory elution with the mobile phase. The final HPLC conditions are as described in section 2.2.1.

Protein samples containing lignocaine *N*-oxide and its metabolites must not be directly injected onto an HPLC column in order to maximise the column life. Various deproteination and extraction schemes, *e.g.* methanol, acetonitrile, chloroform and dichloromethane under acidic or basic conditions, were tested in order to achieve good recovery and prevent compound decomposition. The sample handling procedures used in this study is described in section 2.2.2.

2.2.1 High performance liquid chromatography (HPLC) of lignocaine *N*-oxide and its major metabolites

The HPLC system consisted of a Philips PU4100 liquid chromatograph, a model PU4110 UV/VIS variable-wavelength detector, a model PU4700 autoinjector. Data and chromatograms were recorded. Peak heights and areas were calculated using Jones JCL 6000 data processing software.

The stationary phase consisted of a Jones 5 μm octyl stainless steel cartridge column (4.6 x 150 mm I.D.) and a 5 μm octyl guard column (4.6 x 20 mm I.D.). Chromatography was performed at room temperature using a mobile phase consisting of methanol-acetonitrile-water containing 5 mM 1-heptanesulphonic acid sodium salt, triethanolamine (4% v/v) and acetic acid (4% v/v), pH 4.8 (16 : 16 : 68) at a flow rate of 1.5 ml/min. The UV detector was set at 250 nm.

2.2.2 Sample preparation

In the metabolism experiments, the samples contained 100 μl of freshly prepared NADPH generating system; 50 μl of drug/metabolite solution, 50 μl of biological sample (protein content indicated for each experiment), and 50 μl of 0.01 M

phosphate buffer (pH 7.4) to make a final volume of 250 µl. Any constituent omitted in this sample preparation formula was replaced by 0.01 M phosphate buffer (pH 7.4) to maintain a final incubation volume of 250 µl. Protein concentrations (protein assay as shown in the Appendix I) were measured in biological samples (*e.g.* rat heart and liver subcellular fractions).

In the incubation studies, all samples were deproteinated and extracted by the following method. The 250 µl sample was acidified with 50 µl of 1.0 M hydrochloric acid, and 200 µl of acetonitrile containing disopyramide (12.5 µg/ml), as internal standard, added. The sample was then vortex mixed and chilled thoroughly in ice. Following centrifugation at 1,000 g for 10 min (Jouan, HEMA-C), the supernatant was analysed by HPLC. Peak height ratios (sample peak height/internal standard peak height) or peak area ratios were used to calculate the amount of drug and its metabolites contained in the biological samples. The concentrations of drug and metabolite were quantified from calibration graphs prepared using biological suspensions with known amounts of drug or metabolite added.

In the haem and NADPH supplemented cytochrome P450 and haem oxygenase studies, blanks and samples were incubated anaerobically in a glove box by purging the incubation mixtures with nitrogen and maintaining the incubation mixtures in an atmosphere of nitrogen (see section 2.3.1).

2.2.3 Validation of analytical method

Five calibration standards with known concentrations of lignocaine *N*-oxide (40-200µM) and its presumed metabolites, lignocaine (42-210 µM) and MEGX (49-245 µM), were prepared in rat liver microsomes (protein content, 6.0 mg/ml). These standards were processed by the HPLC sample preparation procedure (see Section 2.2.2) and suitable aliquots injected into the HPLC. Calibration data from these

standard curves were obtained daily, in triplicate, for at least 3 days. Standards were also routinely injected during sample HPLC analysis to evaluate the reproducibility of this system.

Samples of rat liver microsomes (protein content, 6.0 mg/ml), heart S9 fractions (protein content, 10.7 mg/ml), sarcosomes (protein content, 3.7 mg/ml) and cytosol (protein content, 7.5 mg/ml) containing known concentrations of lignocaine *N*-oxide (up to 200 μM), lignocaine (up to 210 μM) and MEGX (up to 245 μM) were prepared following the HPLC sample preparation method and analysed by HPLC (see Section 2.2.2). Recoveries were obtained by comparing the results for each compound in the biological samples with those for standard drug solutions prepared in 0.01 M phosphate buffer (pH 7.4).

2.3 The metabolism of lignocaine N-oxide in rat tissues

2.3.1 Control of oxygen availability

All experiments were performed inside an airtight glove box (690 x 430 x 400 mm). The glove box was evacuated *via* flow meters and the amounts of compressed air and nitrogen were introduced to produce oxygen concentrations from 0 to 20% mg/L (31-625 μmole/L of oxygen). Oxygen concentrations were monitored using a Clarke-type oxygen electrode (Strathkelvin Instruments, Glasgow, U.K., model 781) at the incubation temperature of 37°C, throughout each incubation period. During the incubation studies all solutions used were kept under the desired oxygen concentration before and throughout the experiments. Biological samples were incubated and analysed by HPLC (as described in section 2.2). Each experiment was performed at least in triplicate.

2.3.2 Control studies for lignocaine *N*-oxide metabolism

A series of experiments were carried out to investigate the requirement for each incubation component in lignocaine *N*-oxide metabolism. The constituents of the NADPH generating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $MgCl_2$), the NADPH generating system (NADPH) and rat liver microsomes were each omitted from the total incubation mixture. Anaerobic incubation of lignocaine *N*-oxidation and rat heart homogenate was carried out with and without the addition of NADPH generating system.

The fate of lignocaine (336 μM) with rat liver microsomal suspensions (protein content, 6 mg/ml), and lignocaine *N*-oxide (200 μM) with rat heart S9 suspension (protein content, 2.7 mg/ml) was studied under anaerobic conditions for 90 minutes. Samples were handled and analysed following the standard analytical scheme as described in section 2.2.

2.3.3 Metabolism of lignocaine *N*-oxide under anaerobic conditions

Anaerobic conditions were produced by deaerating the incubation mixtures with nitrogen. Incubation mixtures (250 μl) were NADPH supplemented and prepared as described in section 2.2.1. Samples were incubated anaerobically using a glove box as described in section 2.2.3.1. Lignocaine *N*-oxide (200 μM) was incubated in rat liver microsomal suspensions (protein content, 6 mg/ml) for 50 min. Lignocaine *N*-oxide (200 μM) was incubated for 60 minutes with rat heart subcellular fractions. The fractions used were heart S9 suspension (protein content, 2.7 mg/ml), sarcosomes (protein content, 0.8 mg/ml) and cytosol (protein content, 1.6 mg/ml). All samples collected were handled following the standard analytical scheme as described in section 2.2.

2.3.4 Effect of oxygen tension on lignocaine *N*-oxide reduction

Incubations under various oxygen concentrations from 0 to 20% were performed in a closed system as described in section 2.3.1. Lignocaine *N*-oxide (200 μM) in NADPH supplemented rat liver microsomal suspensions (protein content, 3.6 mg/ml) were incubated under different oxygen tensions for 15 min. Rat heart homogenate (protein content, 2.15 mg/ml) and heart subcellular fraction (S9, protein content, 2.5 mg/ml) were incubated under several different oxygen tensions for 10 min. All samples were handled and analysed as described in section 2.2.

2.3.5 Enzyme kinetics of lignocaine *N*-oxide reduction

The enzyme kinetic constants for the reduction of lignocaine *N*-oxide were determined in rat liver microsomes and heart subcellular fractions. The optimal conditions for *N*-oxide reduction with respect to substrate and protein concentration under anaerobic conditions was investigated.

2.3.5.1 Enzyme kinetics of lignocaine *N*-oxide reduction in rat liver microsomes

Incubation mixtures of rat liver microsomes (protein content, 6 mg/ml) with different concentrations of lignocaine *N*-oxide (40-400 μM) were incubated for 5 min. Different volumes of a standard rat liver microsomal suspension (protein content, 6 mg/ml) were used to vary the protein (enzyme) concentration (60-1270 μg/ml) in 250 μl incubation mixtures containing lignocaine *N*-oxide (200 μM). Five-minute incubations were carried out under anaerobic conditions.

2.3.5.2 Enzyme kinetics of lignocaine *N*-oxide reduction in rat heart subcellular fraction

Different concentrations (30-400 μM) of lignocaine *N*-oxide in rat heart S9 suspension (protein content, 2.36 mg/ml) were incubated for 10 min. Different volumes of a standard rat heart S9 suspension were used to vary the protein (enzyme) concentration (0.47-4.72 mg/ml) in 250 μl incubation mixtures with a fixed lignocaine *N*-oxide concentration of 200 μM. Incubations were carried out under anaerobic conditions for 10 min. All incubation mixtures were prepared and handled according to the standard procedure as described in section 2.2 and analysed by HPLC.

2.3.6 *In vivo* metabolism of lignocaine *N*-oxide in the rat

An intraperitoneal injection of lignocaine *N*-oxide (10 mg/kg) was given to a male rat (weight, 307 g). Urine samples were collected for 24 hours from this rat and a non-dosed control rat (weight, 320 g). Pooled rat urine samples were filtered through 2 μm sample filters, and analysed by HPLC following sample preparation as described in section 2.2. Lignocaine *N*-oxide was added to a sample of blank urine and the solution was left for 24 hours at room temperature.

2.3.7 Metabolism of lignocaine *N*-oxide in isolated perfused rat heart

Metabolism of lignocaine *N*-oxide was studied using perfused rat hearts. Whole hearts from male rats (Sprague-Dawley, 6-12 months) were perfused *via* a Langendorff preparation. The perfusion medium was Tyrode's solution, gassed with O_2: CO_2 (95:5%) at 37 °C and the perfusion rate was 16 ml/min. Freshly prepared rat

hearts (weighing 2.5-3.0 g), were placed in the apparatus and stabilised by perfusion for 15 min. Further perfusion of either Tyrode's solution, lignocaine *N*-oxide (500 μM) or lignocaine (500 μM) in Tyrode's solution, were then continued for 10 min. Perfusion was then stopped for 10 min to achieve anoxic conditions. The perfusion fluids collected from the hearts were collected for analysis. The perfused rat heart was rapidly frozen in liquid nitrogen to prevent any further metabolism. The whole heart was weighed and homogenised at 0-5 °C (in ice) and the homogenates were then diluted with 0.01 M phosphate buffer (pH 7.4) to make a suspension containing 0.2 g heart tissue /ml. All samples were maintained at 0 °C and analysed immediately by HPLC.

2.3.8 Lignocaine *N*-oxide metabolism in cytochrome P450 phenotyped human liver microsomes

Samples containing 50 μl phenotyped human liver microsomes (approximate 0.3 mg/ml), NADPH (1 mM) and lignocaine *N*-oxide (100 μM) in phosphate buffer (pH 7.4, 0.1 mM) with a final volume of 250 μl were incubated anaerobically at 37 °C for 20 min. All samples were processed and analysed according to the standard procedure as described in section 2.2 and analysed by HPLC.

2.4 Mechanistic studies on N-oxide bioreduction

2.4.1 Effect of enzyme inhibitors on lignocaine *N*-oxide reduction

All incubation mixtures contained NADPH supplemented tissue (rat liver microsomes or heart S9 fractions), lignocaine *N*-oxide (200 μM) and were maintained under anaerobic condition as described in section 2.2. In selected incubations either carbon monoxide was added for 60 seconds, or KCN (0.1 mM) added or lignocaine (200 μM) added or functional tissue replaced with denatured (boiled) tissue. Following incubation for 20 min all samples were processed and analysed by HPLC as described in section 2.2.

2.4.2 Visible light difference-spectrum studies of lignocaine *N*-oxide binding to rat liver microsomes

Visible light difference spectroscopy was used to study the binding of lignocaine and lignocaine *N*-oxide to microsomal cytochrome P450 under oxidised or reduced conditions. Spectroscopic examination of microsomal suspensions (0.2 mg/ml) was carried out under air (oxidised microsomes) and under reduced conditions. Reduced microsomal suspensions were prepared by adding 500 mg of sodium dithionite ($Na_2S_2O_4$) to 5 ml of microsomal suspensions. Volumes (50 μl) of lignocaine or lignocaine *N*-oxide stock solutions (4 mM) in 0.01 M phosphate buffer (pH 7.4) were added to 2.0 ml of either oxidised or reduced microsomal suspensions. In all microsomal suspensions, the final drug concentrations were between 0 and 16 μM. The spectra were recorded between 340-500 nm using a double beam spectrophotometer, Perkin Elmer 550S. The reference solutions, consisting of

appropriately treated microsomal suspensions, were as described above, without the addition of drug solutions. All experiments were performed at room temperature.

2.4.3 Effect of cobalt treatment of rats on *in vitro* lignocaine *N*-oxide reduction

Three male rats (Sprague-Dawley, weighing about 500 g) were each given two subcutaneous injections of cobalt chloride (60 mg/kg) at 24 hour intervals. The rats were then starved for 24 hours and killed by decapitation. The cytochrome P450 and b_5 levels in the liver microsomal suspensions of the three dosed rats and a control rat were measured as described in the Appendix II. Lignocaine *N*-oxide (200 μM) was incubated in NADPH supplemented dosed and control rat liver microsomal suspensions (6.8 mg/ml) for 40 min under anaerobic conditions as described in section 2.2 and analysed by HPLC.

2.4.4 Involvement of haem, haem oxygenase, cytochrome P450 reductase and xanthine oxidase in lignocaine *N*-oxide reduction

2.4.4.1 Cytochrome P450 reductase and haem oxygenase

Samples containing lignocaine *N*-oxide (100 μM), purified rat liver cytochrome P450 reductase (3 μl, specific activity 3 nmole NADPH reduced per min), haem oxygenase (2 μl, specific activity 0.7 nmole biliverdin formed per min), haemin (40 μM) and NADPH (200 μM) in 0.01 M phosphate buffer (pH 7.4) in a final volume of 100 μl were incubated anaerobically for 60 min. Studies were also carried out omitting NADPH, haem, haem oxygenase or cytochrome P450 reductase.

2.4.4.2 Xanthine oxidase

Incubation mixtures (final volume 250 μl) containing lignocaine *N*-oxide (100 μM), both milk xanthine oxidase (1 unit) and NADPH (200 μM) in 0.01 M phosphate buffer (pH 7.4) were incubated anaerobically with and without the addition of haemin (40 mM) for 60 min.

In all the above procedures, the incubates were analysed by HPLC following deproteination and extraction as described in section 2.2. Suitable blank incubates were analysed by HPLC in the same manner and at the appropriate time.

2.4.5 Reduction of lignocaine *N*-oxide by myoglobin, haemoglobin and viable myocytes

Deoxymyoglobin and metmyoglobin prepared as described in section 2.1 in 1.0 M Tris-HCl buffer (pH 7.4) were diluted with 0.01 M phosphate buffer (pH 7.4) to obtain protein contents equivalent to 1 mg/ml. Deoxyhaemoglobin and methaemoglobin (protein content 1mg/ml) were diluted with 0.01 M phosphate buffer (pH 7.4) to make protein contents equivalent to 1 mg/ml. Myocytes were prepared in 0.01 M phosphate buffer (pH 7.4) with 10^6 cells per sample. Sample solutions were incubated under anaerobic conditions with lignocaine *N*-oxide (100 μM) for 2 hours. The total volume of the incubation mixture was 250 μl. All solution used were carefully degassed under nitrogen to ensure fully anaerobic conditions. Samples were processed and analysed by HPLC immediately as described in section 2.2.

2.4.6 Influence of oxygen tension on lignocaine *N*-oxide reduction by myoglobin

Under carefully controlled conditions as described in section 2.3, samples containing deoxymyoglobin (protein content, 1 mg/ml) and lignocaine *N*-oxide (100 μM) in a final volume of 250 μl were incubated under various oxygen concentrations for 2 hr. The oxygen content in this system was varied from between 0 and 20%. Samples were processed and analysed by HPLC as described in section 2.2.

2.4.7 Reduction of lignocaine *N*-oxide by inorganic iron and haem

Lignocaine *N*-oxide (100 μM) was incubated anaerobically for 60 min with freshly prepared ferric chloride (Fe^{+3}, 1 mM), ferrous sulphate (Fe^{+2}, 1 mM) and haemin (1 mM) with or without the addition of EDTA (metal chelating agent, 1mM) in 0.01 M phosphate buffer (pH 7.4). Anaerobic incubations (60min) of lignocaine *N*-oxide and haemin with or without the addition of ascorbic acid (1 mM) and NADPH (prepared from NADPH generating system as described in section 2.1.2) were carried out to investigate *N*-oxide reduction by reduced and oxidised haem. Incubations with only NADPH and ascorbic acid were performed as control studies. Samples were processed and analysed by HPLC as described in section 2.2.

2.4.8 Interaction of lignocaine *N*-oxide with iron and myoglobin as determined by electron spin resonance spectrometry

In the mechanism studies of *N*-oxide reduction, electron spin resonance (ESR) spectrometry was used to detect lignocaine *N*-oxide reduction. A Varian E3 X-band electron spin resonance (ESR) spectrometer was used and operated at room temperature. The operating conditions were: microwave power 10 mW; microwave frequency 9.44 G; magnetic field 3395 G; modulation amplitude 1.25 G; receiver gain 3.2×10^5; scan range 50 G; time constant 0.3 second and scan time 4 min. The quartz flat cells and all components used were checked individually to ensure that no interfering ESR signals occurred. A spin trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, 1 mM) was added to samples.

Electron spin resonance spectra of ferrous iron ($FeSO_4$, 1 mM), deoxymyoglobin (50 μg), metmyoglobin (50 μg) and ferryl myoglobin (50 μg) were recorded in the presence of lignocaine (1 mM), lignocaine *N*-oxide (1 mM), methylamine (250 μl) hydrogen peroxide (H_2O_2, 100 mM), EDTA (1mM), superoxide dismutase (1 unit), catalase (1 unit) or DMSO (250 μl). A series of experiments were carried out using ESR spectroscopy to detect possible free radical formation from the interactions of lignocaine *N*-oxide and myoglobin (Table 2-1). All samples were prepared in 0.01 M phosphate buffer (pH 7.4) containing a spin trapping agent, DMPO (100 mM) and a final volume of 500 μl. All experiments were performed under anaerobic conditions unless specifically indicated.

Table 2-1 Experimental conditions, components added and their concentrations in the electron spin resonance spectroscopy studies of lignocaine *N*-oxide

Component added	Experimental condition															
	Anaerobic											Aerobic				
DMPO (1mM)	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
FeSO4 (1mM)														√	√	√
EDTA (1mM)														√	√	√
Hydrogen peroxide (100mM)							√							√	√	√
Deoxymyoglobin (50mg)	√	√	√	√	√							√	√			
Metmyoglobin (50mg)						√	√									
Ferryl myoglobin (50mg)								√	√	√	√	√				
Lignocaine (1mM)				√						√		√				
Lignocaine *N*-oxide (1mM)		√	√			√					√		√			
DMSO (250mg)									√						√	
Methylamine (250ml)					√											
SOD (1 unit) +catalase (1 unit)			√													√

Experimental details were as described in section 2.4.8.

√ Components added.

2.4.9 Spectral binding studies of lignocaine *N*-oxide with myoglobin

UV/visible light difference spectroscopy was used to study lignocaine *N*-oxide reduction in the presence of myoglobin. Myoglobin was prepared as described in section 2.1. Myoglobin solutions were adjusted to pH 7.4 with 0.1 M hydrochloric acid and fully degassed with nitrogen before use. Experiments were subsequently carried out under anaerobic conditions. The reference solutions consisted of appropriate myoglobin solutions without the addition of lignocaine *N*-oxide.

The binding of lignocaine *N*-oxide (12.5 mM) and lignocaine (12.5 mM) with deoxymyoglobin, metmyoglobin and ferryl myoglobin (all myoglobin protein concentrations were 0.5 mg/ml) were examined. The spectral changes that occurred between 200 and 600 nm were recorded using a double beam spectrophotometer (Perkin Elmer, 550S). Lignocaine *N*-oxide was incubated with myoglobin at 37°C for 60 min under anaerobic conditions.

Solutions containing deoxymyoglobin (0.45 mg/ml) were incubated anaerobically with lignocaine *N*-oxide (1 mM), amiodarone *N*-oxide (1 mM), diltiazem *N*-oxide (1 mM) or disopyramide *N*-oxide (1 mM) for 2.5 hr at 37 °C. Difference UV/visible spectra were recorded on a double beam spectrophotometer (Perkin Elmer, 550S) at intervals during incubation.

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Chapter 3 Results

3.1 HPLC analysis of lignocaine *N*-oxide and metabolites

The chromatographic method developed allowed the analysis of lignocaine *N*-oxide, lignocaine and monoethylglycinexylidide (MEGX), the major metabolite of lignocaine in rat liver microsomes[36]. Analysis of suitable control samples showed that the biological material *per se* did not produce interfering peaks.

Typical chromatograms obtained from the HPLC analysis of rat liver microsomal suspensions spiked with authentic lignocaine *N*-oxide, lignocaine and MEGX are shown in Figure 3-1. HPLC chromatograms of rat heart S9 fraction, sarcosome and cytosol containing lignocaine *N*-oxide, lignocaine and MEGX are shown in Figure 3-2. The retention times were MEGX (6.0 min), lignocaine *N*-oxide (7.5 min), lignocaine (8.5 min) and disopyramide (12.8 min).

Table 3-1 and Figure 3-3 show the calibration data for analysis of lignocaine, lignocaine *N*-oxide and MEGX in rat liver microsomal suspensions. The square correlation coefficient (r^2) for lignocaine (42-210 μM), lignocaine *N*-oxide (40-200 μM) and MEGX (49-246 μM) in the presence of rat liver microsomes are all greater than 0.998. The method was shown to be accurate and precise through the low variation of inter- and intra- experimental results. The day-to-day reproducibility of the assay is shown in Table 3-2. The coefficient of variation (C.V., %) values for both retention times and assay results were calculated, with all values being less than 5%.

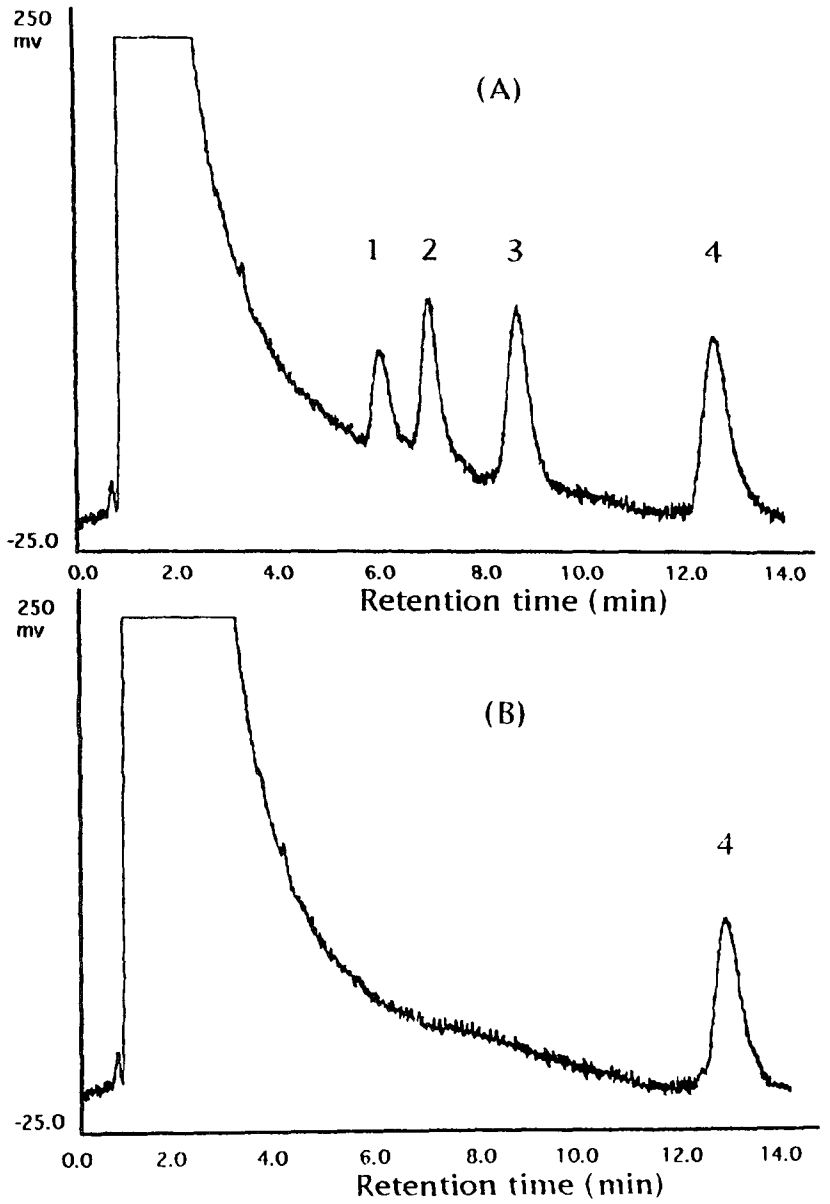


Figure 3-1 HPLC chromatograms of (A) rat liver microsomes containing : (1) monoethylglycinexylidide (2) lignocaine *N*-oxide (3) lignocaine and (4) disopyramide (internal standard); (B) rat liver microsomes containing internal standard.

*HPLC conditions were as described in section 2.2.1.

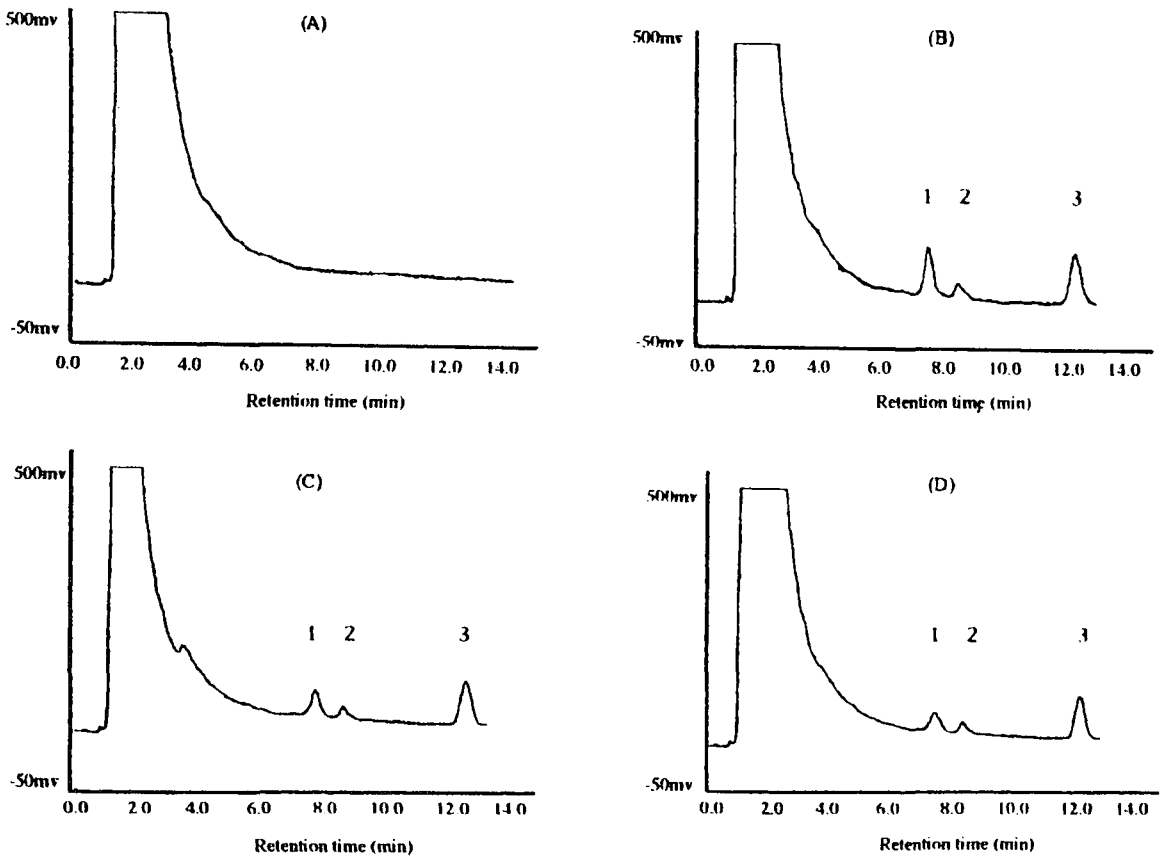


Figure 3-2 HPLC chromatograms of (A) blank rat heart subcellular fractions (S9) and (B) rat heart S9 fractions (C) sacrosomes (D) cytosolic suspension containing: (1)lignocaine *N*-oxide (2) lignocaine and (3) disopyramide (internal standard).

HPLC conditions were as described in section 2.2.1.

Table 3-1 HPLC calibration data for lignocaine *N*-oxide, lignocaine and monoethylglycinexylidide in the presence of rat liver microsomes

Compound	Concentration (μm)	Peak height ratio	Calibration curve
lignocaine *N*-oxide	40	0.7303±0.0152	
	80	1.3763±0.0092	
	120	1.9520±0.0614	
	160	2.6283±0.0372	Y=0.0508±0.0160X
	200	3.2331±0.0840	r^2=0.999
lignocaine	42	0.3482±0.0072	
	84	0.6752±0.0143	
	126	0.9641±0.0235	
	168	1.2493±0.0187	Y=0.0239±0.0074X
	210	1.5849±0.0261	r^2=0.999
MEGX	49	0.3172±0.0140	
	98	0.5911±0.0186	
	147	0.9005±0.0214	
	196	1.1646±0.0560	Y=0.0253±0.0057X
	245	1.3996±0.0206	r^2=0.998

HPLC analysis was performed as described in section 2.2.3.

All results are the mean ±standard deviation of three replicates.

MEGX= monoethylglycinexylidide

Peak height ratio= analyte peak height/internal standard peak height

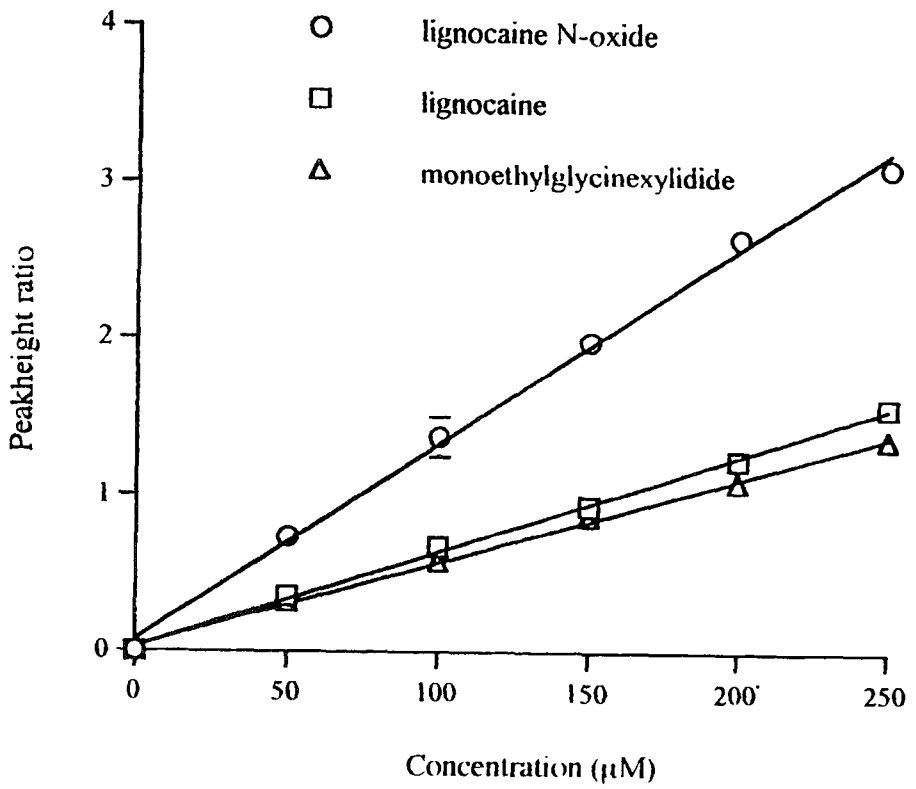


Figure 3-3 Calibration graph for lignocaine *N*-oxide, lignocaine and monoethylglycinexylidide in rat liver microsomal suspensions. Results are the mean ± s.d. of three determinations, see section 2.2.3 for experimental details.

Table 3-2 Inter day variation of analysis of lignocaine *N*-oxide,lignocaine and monoethylglycinexylidide in the presence of rat liver microsomes

Compound	Concentration (μM)		CV (%)
	added	found	
lignocaine N-oxide	40	39.92 ± 0.19	0.48
	80	79.77 ± 0.24	0.30
	120	121.31 ± 1.43	1.18
	160	161.54 ± 3.52	2.18
	200	200.02 ± 7.53	3.76
lignocaine	42	40.92 ± 0.32	0.77
	84	83.76 ± 2.42	2.89
	126	125.17 ± 1.45	1.16
	168	169.42 ± 0.67	0.40
	210	211.02 ± 2.58	1.22
MEGX	49	50.00 ± 2.25	4.50
	98	97.98 ± 1.11	1.13
	147	145.93 ± 5.25	3.60
	196	197.71 ± 8.05	4.07
	245	248.10 ± 9.15	3.69

HPLC analysis was performed as described in section 2.2.3.

All results are the mean ± standard deviation of three replicates.

Coefficient of variation (CV) = standard deviation/mean (%).

MEGX= monoethylglycinexylidide

It has been noticed that in this study, a quantification error occurred when extra-plotting lignocaine *N*-oxide below the concentration of 50 μM. The amount of lignocaine *N*-oxide was found to be lower than the actual amount found. A different standard curve at the range between 0 and 50 μM should be used in the quantification of lignocaine *N*-oxide, lignocaine or MEGX to provide precise quantification. In this study, no other metabolite except lignocaine has been found. The formation of lignocaine is therefore considered to be quantitatively equivalent to the metabolism of lignocaine *N*-oxide.

The recoveries of lignocaine *N*-oxide and lignocaine in rat liver microsomal suspensions, rat heart subcellular fractions (S9, sarcosome and cytosol) were all greater than 92%; the recovery of MEGX was greater than 82% (as shown in Table 3-3). The lower recoveries of lignocaine and MEGX than lignocaine *N*-oxide may be due to the extraction system used being more suitable for extracting polar compounds such as lignocaine *N*-oxide rather than the more lipophilic protein bound drugs and metabolites.

3.2 Metabolism of lignocaine *N*-oxide in rat liver and heart

Lignocaine *N*-oxide was converted to lignocaine in anaerobic rat liver microsomes supplemented with NADPH (see Figure 3-4). When lignocaine *N*-oxide was incubated under anaerobic conditions, no reduction occurred in the absence of NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $MgCl_2$, the NADPH generating system and microsomes, respectively. Additionally, lignocaine *N*-oxide did not react with any single one of these components upon anaerobic incubation. In the rat heart homogenate, no *N*-oxide reduction occurred in the anaerobic incubation without the supplement of NADPH (from the NADPH generating system).

Table 3-3 Intra batch recoveries of lignocaine *N*-oxide, lignocaine and monoethylglycinexylidide in the presence of rat tissue fractions.

Cell fraction	Protein content (mg/ml)	Recovery (%)		
		lignocaine N-oxide	lignocaine	MEGX
rat liver microsomes	6.0	97.09±0.46	97.07±0.25	85.42±1.36
rat heart s9 fraction	10.7	96.21±0.21	96.21±0.21	82.64±0.86
rat heart sarcosomes	3.7	94.50±0.36	92.13±0.31	84.90±0.75
rat heart cytosol	7.5	97.33±0.68	93.07±0.63	87.42±0.51

Sample preparation and HPLC analysis were as described in section 2.2.

Tissue fractions were prepared as described in section 2.1.3.

All results are the mean ± standard deviation of three replicates.

MEGX= monoethylglycinexylidide

$$\text{Recovery (\%)} = \frac{\text{peak height ratio of analyte in cell fraction}}{\text{peak height ratio of analyte in buffer}}$$

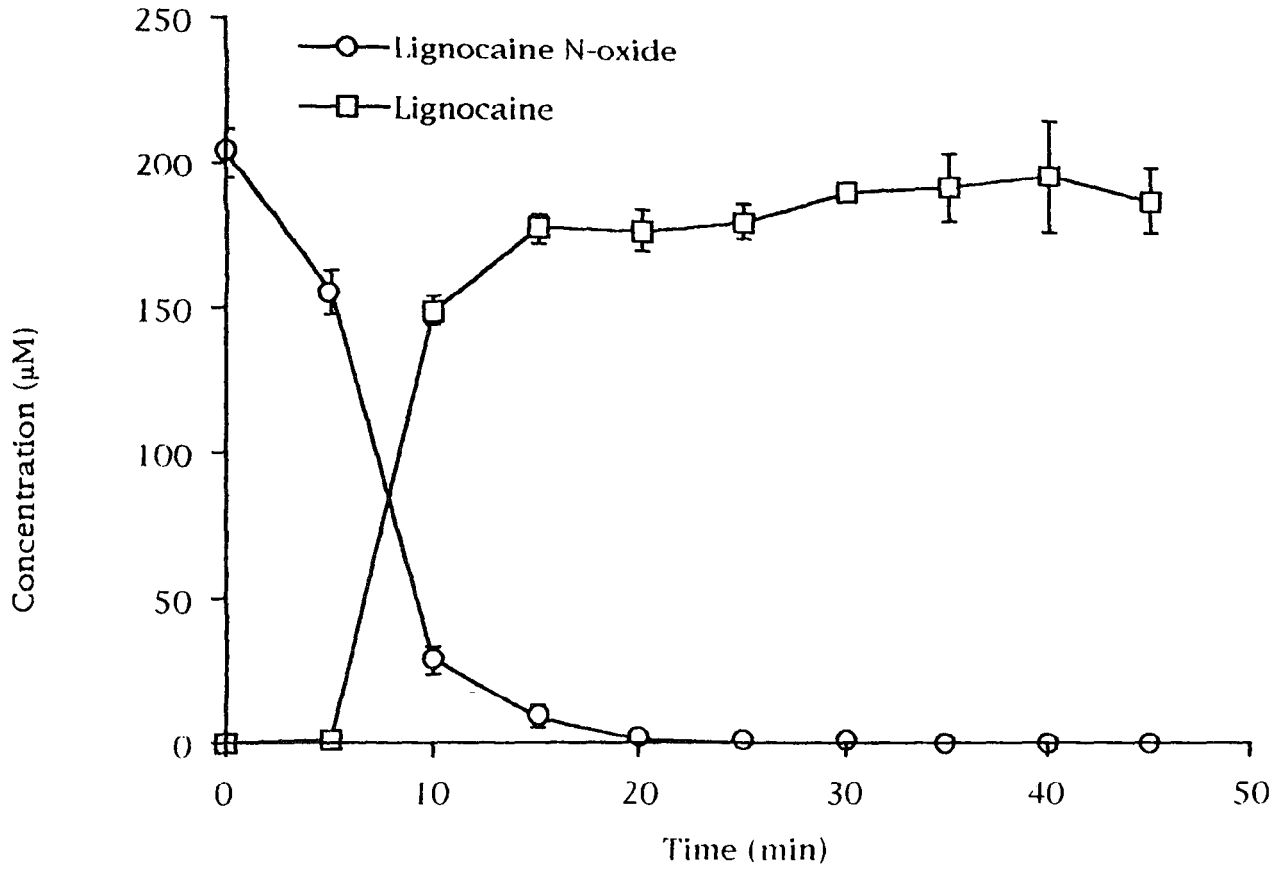


Figure 3-4 Reduction of lignocaine *N*-oxide by NADPH supplemented rat liver microsomes under anaerobic condition. Experimental details as described in section 2.3. Results are the mean ± s.d. of three determinations.

Lignocaine remained unchanged under anaerobic incubation with rat liver microsomes and rat heart homogenate for at least 90 min (Figure 3-5). This suggests that when lignocaine *N*-oxide was incubated in this system, any lignocaine formed by *N*-oxide reduction would not be oxidised back to lignocaine *N*-oxide or be converted/hydrolysed into any other metabolite under the conditions employed.

3.2.1 Metabolism of lignocaine *N*-oxide in rat liver and heart tissue

Lignocaine *N*-oxide and lignocaine were the only substances detected when lignocaine *N*-oxide was incubated, under anaerobic conditions, with either rat liver microsomes, rat heart S9 fractions or rat heart cytosol. This together with quantitative recovery indicates that no other metabolites were found under the conditions used. Lignocaine *N*-oxide metabolism was complete within 60 min in rat liver microsomes, rat heart S9 fractions and cytosolic fractions (Figure 3-4, 3-6 and Figure 3-7). Incubation of lignocaine *N*-oxide with sarcosomes did not result in any detectable formation of lignocaine in incubations up to 60 min (Figure 3-8).

When lignocaine *N*-oxide was incubated with rat liver microsomal suspension under diminished oxygen tension, an decrease in reduction to lignocaine was observed proportional to the oxygen content (see Figure 3-9). The formation of lignocaine was in proportion to the loss of lignocaine *N*-oxide. Lignocaine *N*-oxide reduction was suppressed when the oxygen concentration was increased but was not fully inhibited under aerobic conditions. Specifically under anaerobic conditions, more than 60% of the *N*-oxide was reduced to lignocaine whilst under aerobic condition less than 16% was reduced. From the chromatograms, it appeared that lignocaine was the only metabolite formed.

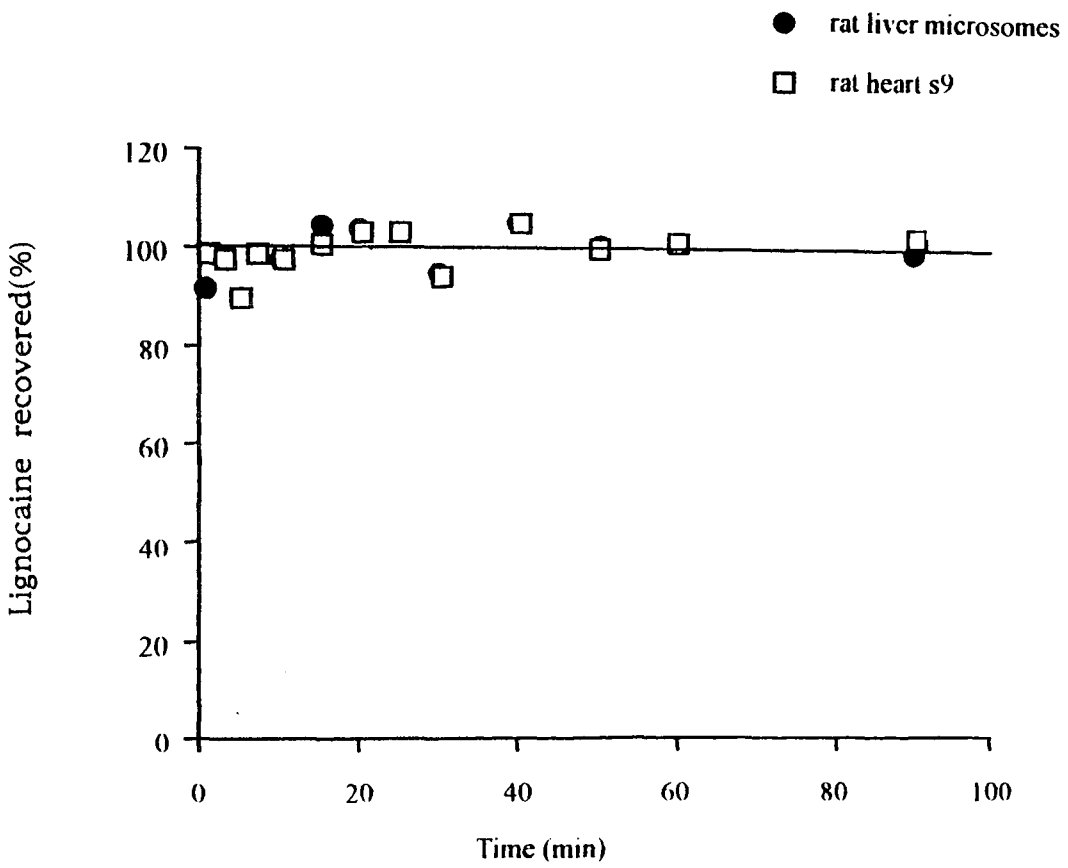


Figure 3-5 Anaerobic incubation of lignocaine with rat liver microsomes and rat heart S9 fractions. Experimental details were as described in section 2.3.2.
Results are the mean ± s.d. of three determinations.

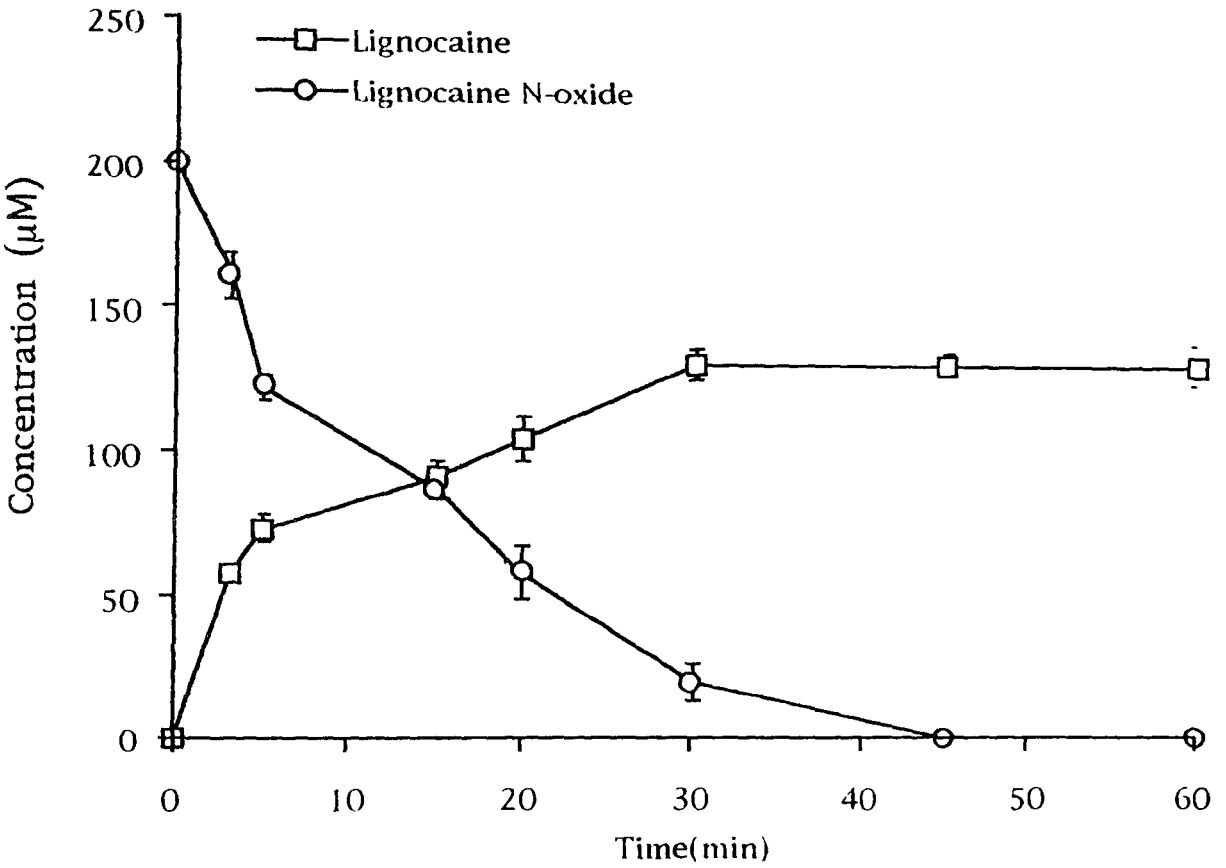


Figure 3-6 Reduction of lignocaine *N*-oxide by NADPH supplemented rat heart S9 fractions under anaerobic conditions. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

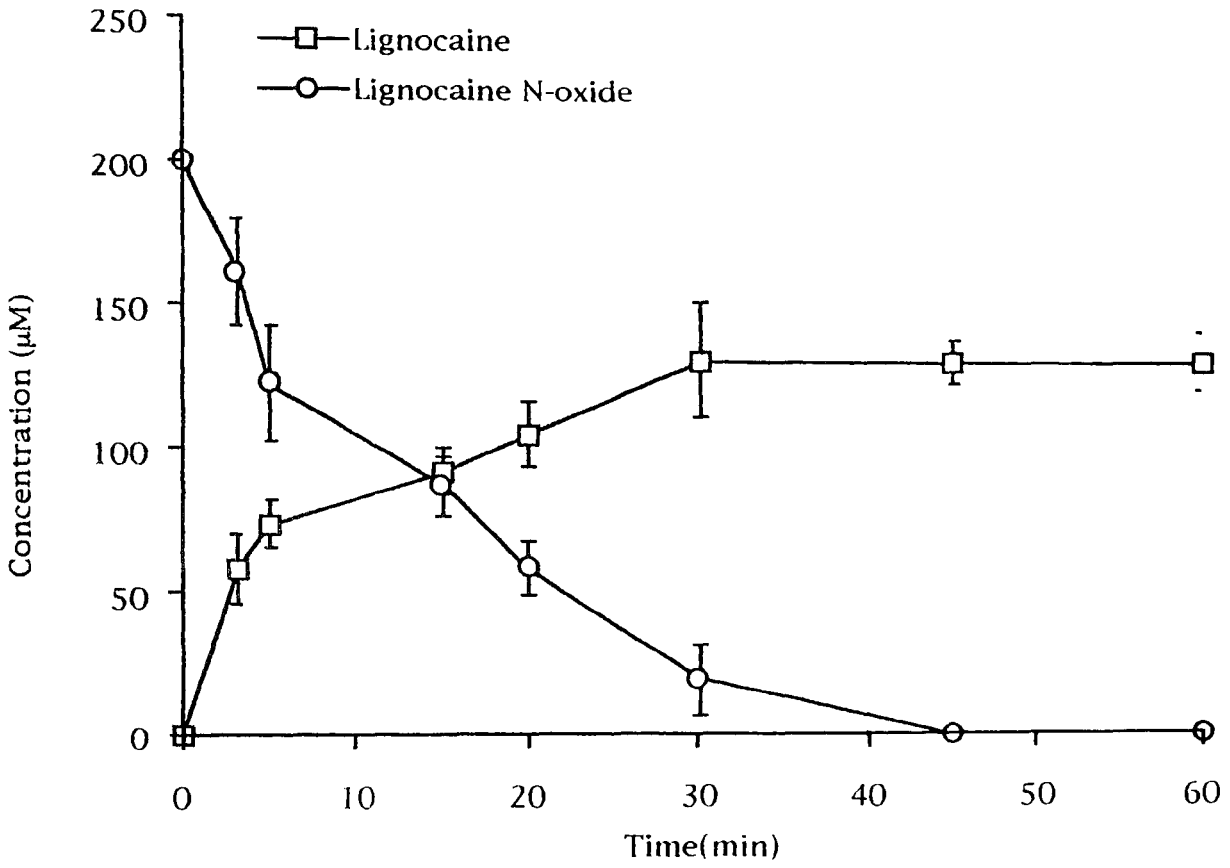


Figure 3-7 Reduction of lignocaine *N*-oxide by NADPH supplemented rat heart cytosol under anaerobic condition. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

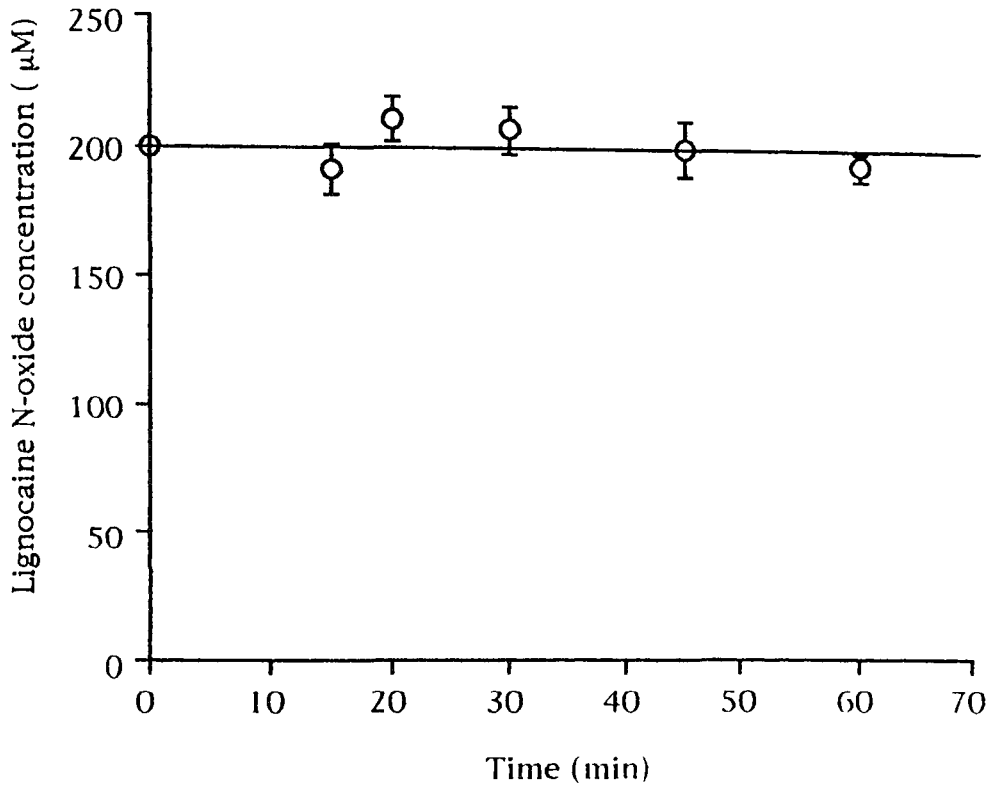


Figure 3-8 The anaerobic incubation of lignocaine *N*-oxide in rat heart sarcosomes . Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

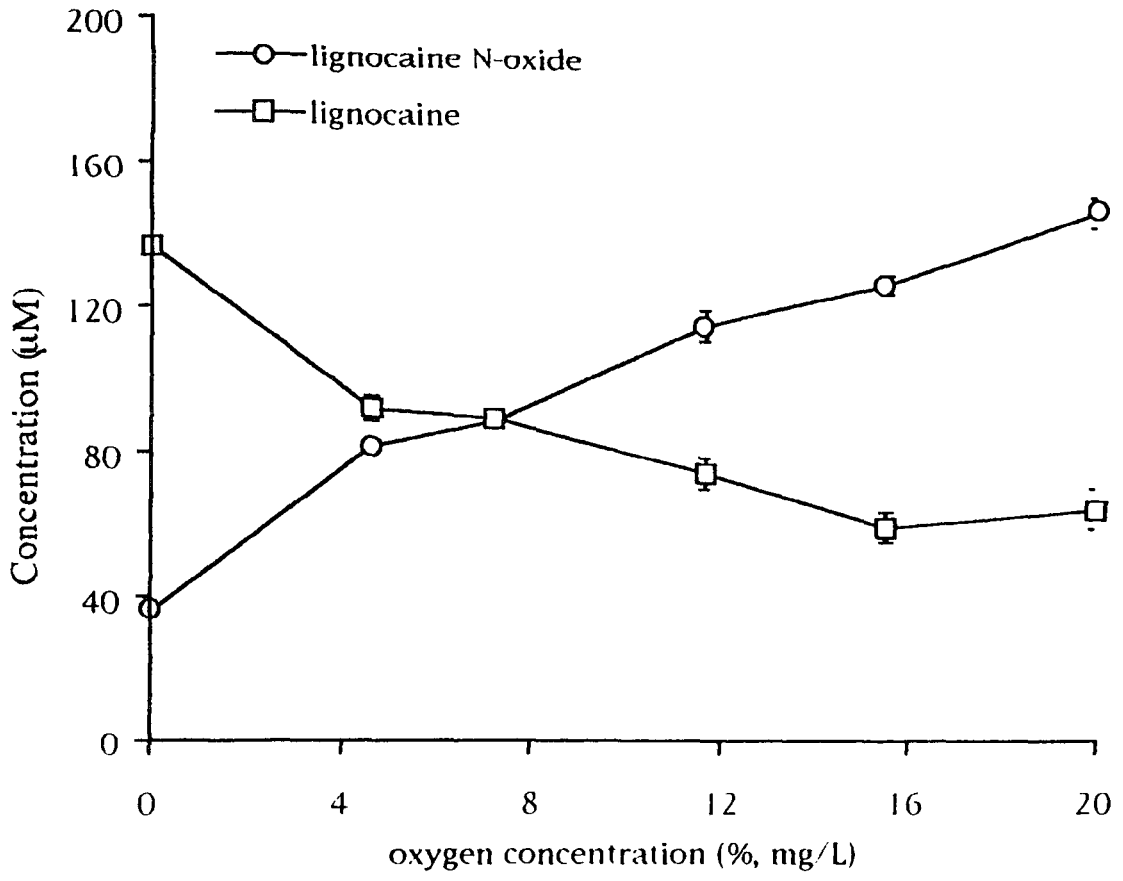


Figure 3-9 Effect of oxygen tension on lignocaine *N*-oxide reduction in NADPH supplemented rat liver microsomal suspensions. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations. 1 mg/L oxygen = 31.3 mmole /L oxygen.

As shown in Figure 3-10 and Figure 3-11, lignocaine *N*-oxide reduction was also inhibited by oxygen in both rat heart homogenate and S9 fractions. Lignocaine production under anaerobic conditions in homogenate and S9 was greater than 75% whilst less than 23% was converted into lignocaine under aerobic conditions.

3.2.2 Enzyme kinetics of lignocaine *N*-oxide metabolism

The enzyme concentration was controlled by adjusting the protein content in the rat liver microsome incubation mixtures. As the enzyme content increased, there was an increase in the amount of lignocaine *N*-oxide metabolised and concomitant formation of lignocaine (Figure 3-12). The effect of substrate concentrations on lignocaine *N*-oxide reduction in rat liver microsomes is shown in Figure 3-13. From this data, enzyme kinetic constants (Km and Vmax) for lignocaine *N*-oxide reduction were determined using the Lineweaver-Burke linear transformation. These plots are shown in Figure 3-14 and the values for Km and Vmax were determined to be 250.0 μM and 0.992 nmole/min/mg protein, respectively.

The effect of enzyme concentration on lignocaine *N*-oxide metabolism in heart S9, cytosol and sarcosomes is shown in Figure 3-15. Lignocaine *N*-oxide reduction increased as protein content increased. The effect of substrate concentration on lignocaine *N*-oxide reduction in S9 fraction is shown in Figure 3-16. The enzyme kinetic constants (Km and Vmax) for lignocaine *N*-oxide reduction were calculated from Figure 3-17 and found to be 269.8 μM for Km and 1.11 nmole/min/mg protein for Vmax.

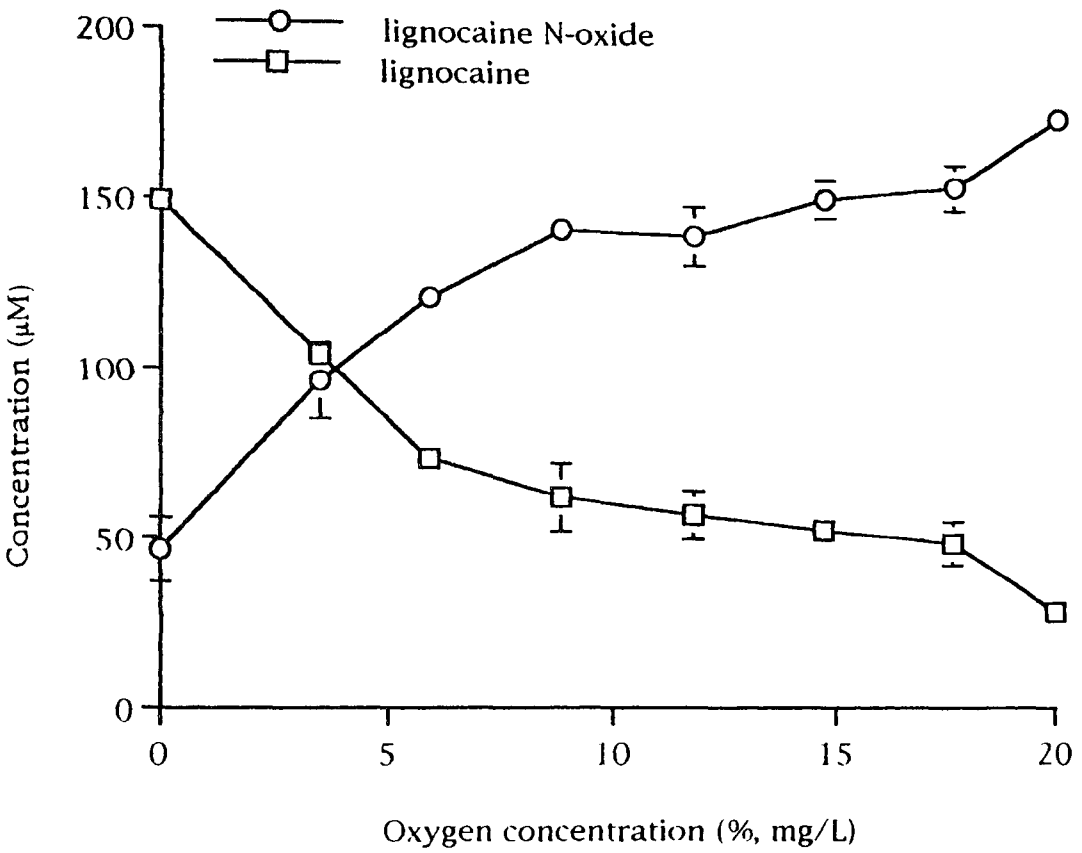


Figure 3-10 Effect of oxygen tension on lignocaine *N*-oxide reduction in NADPH supplemented rat heart homogenate. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

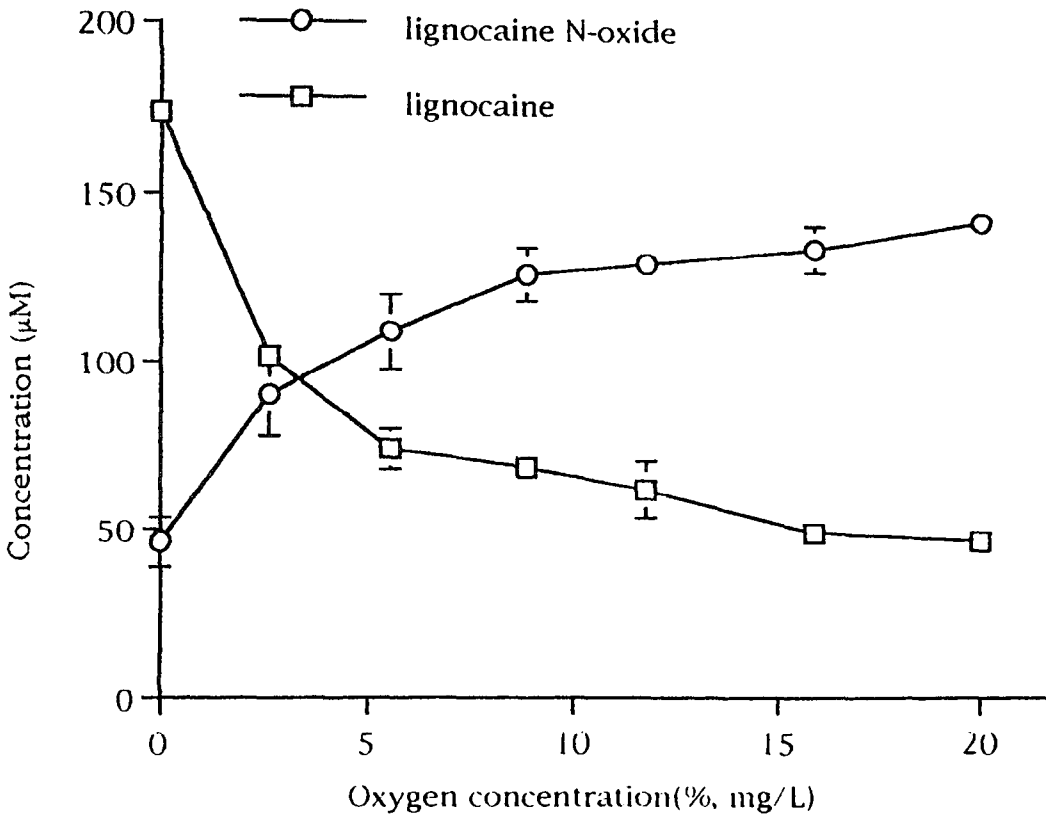


Figure 3-11 Effect of oxygen tension on lignocaine *N*-oxide reduction in NADPH supplemented rat heart *S9* fractions. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

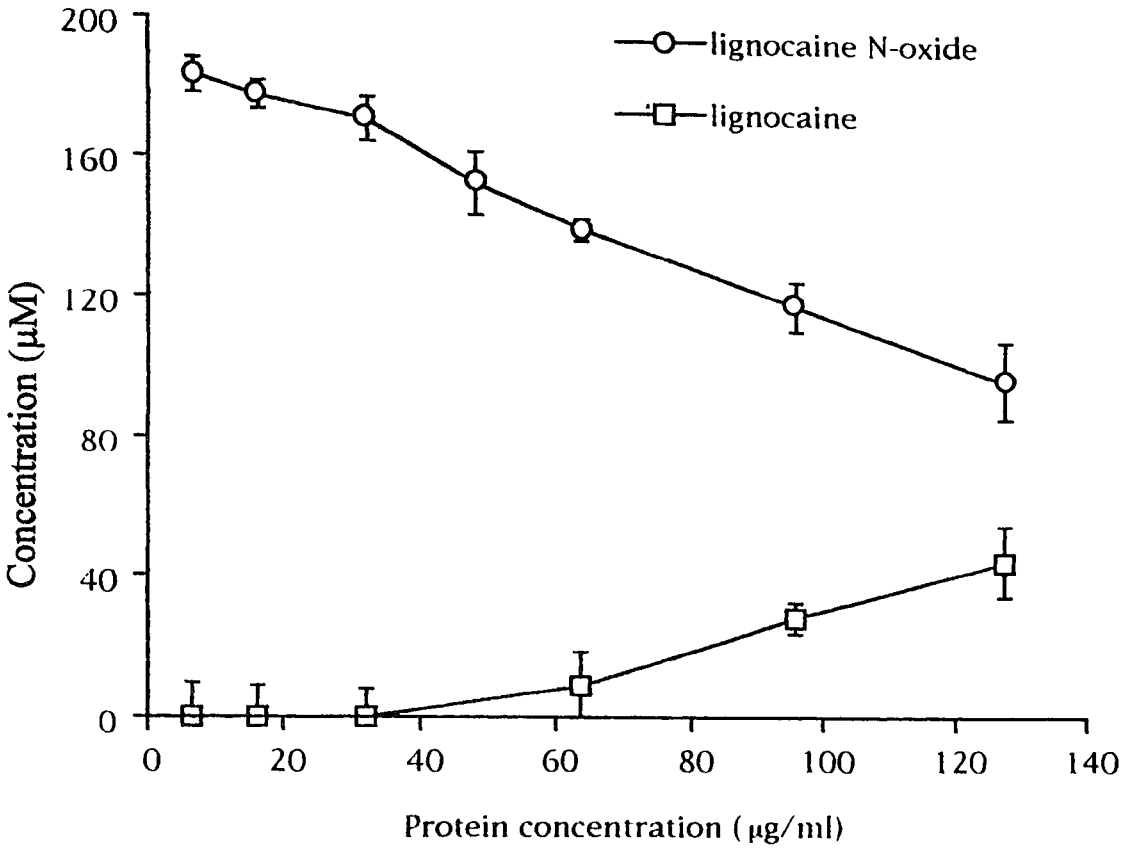


Figure 3-12 Effect of NADPH supplemented rat liver microsomal protein concentration on lignocaine *N*-oxide reduction under anaerobic conditions. Experimental details were as described in section 2.3.
Results are the mean ± s.d. of three determinations.

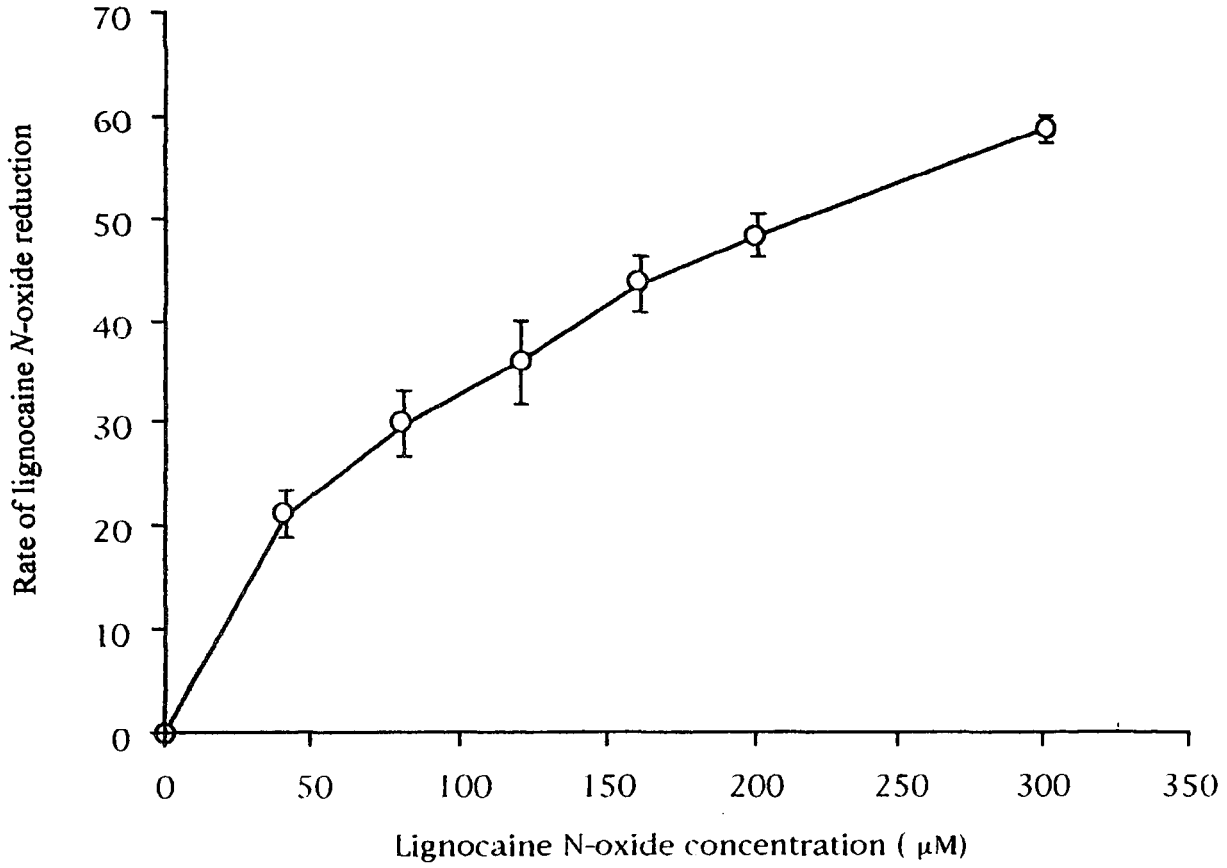


Figure 3-13 Effect of lignocaine *N*-oxide concentration on its reduction in NADPH supplemented rat liver microsomal suspensions under anaerobic condition. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

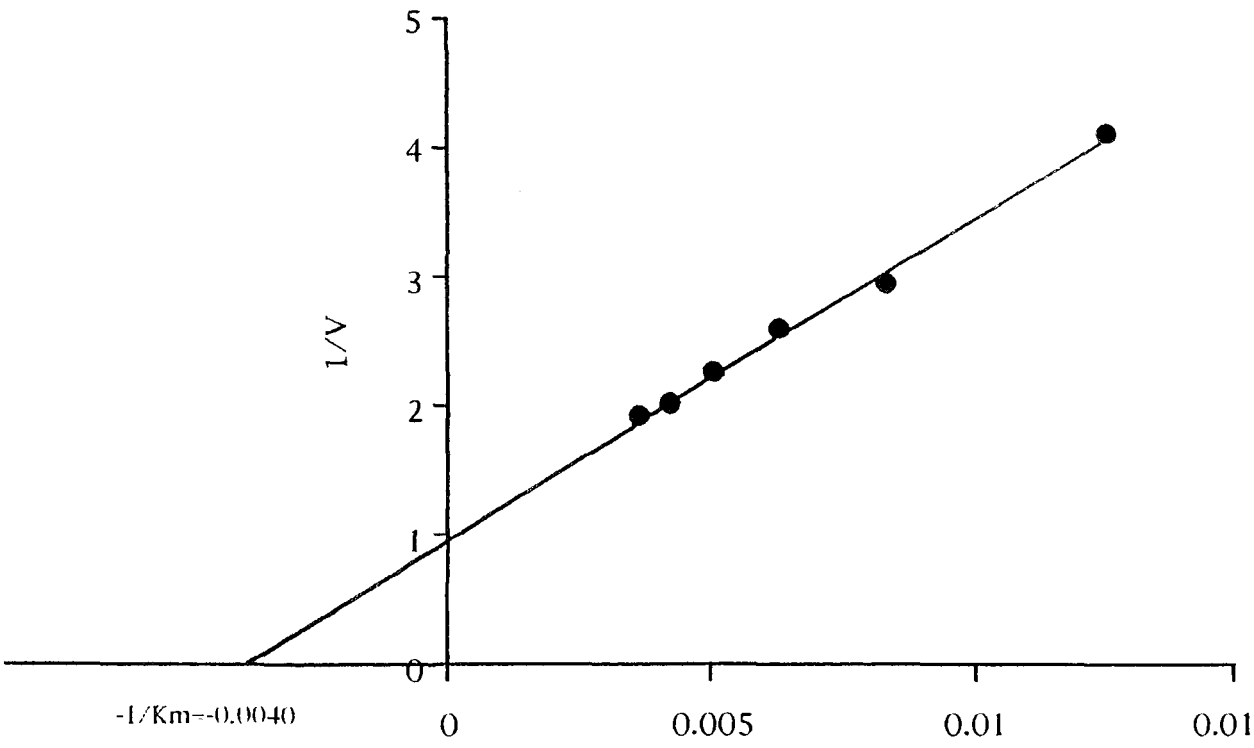


Figure 3-14 Lineweaver-Burk plot for lignocaine *N*-oxide reduction in NADPH supplemented rat liver microsomes. Experimental details as described in section 2.3.

Linear regression equation : Y = 1.0082 + 250.1 X
Correlation coefficient (r) = 0.9920
Km= 250.0 μM
Vmax = 0.9920 nmole/min/mg

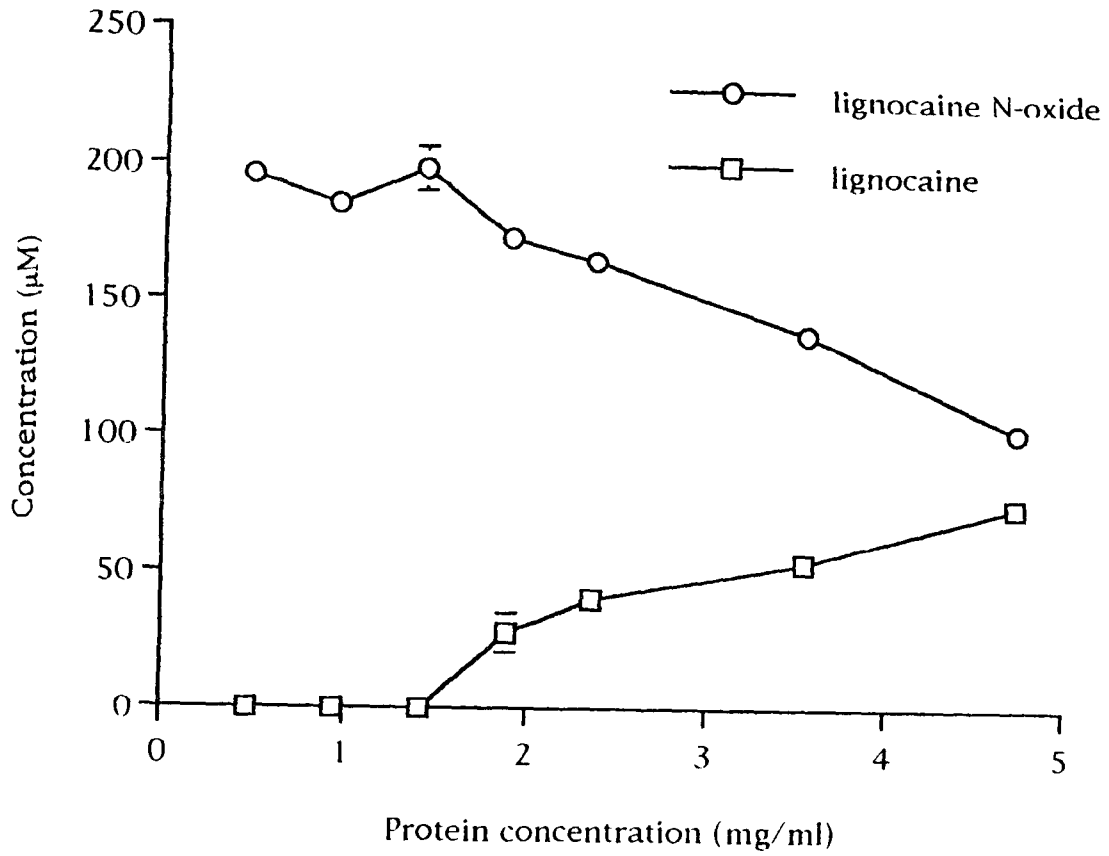


Figure 3-15 Effect of NADPH supplemented protein concentration of rat heart *S9* fraction on lignocaine reduction under anaerobic conditions. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

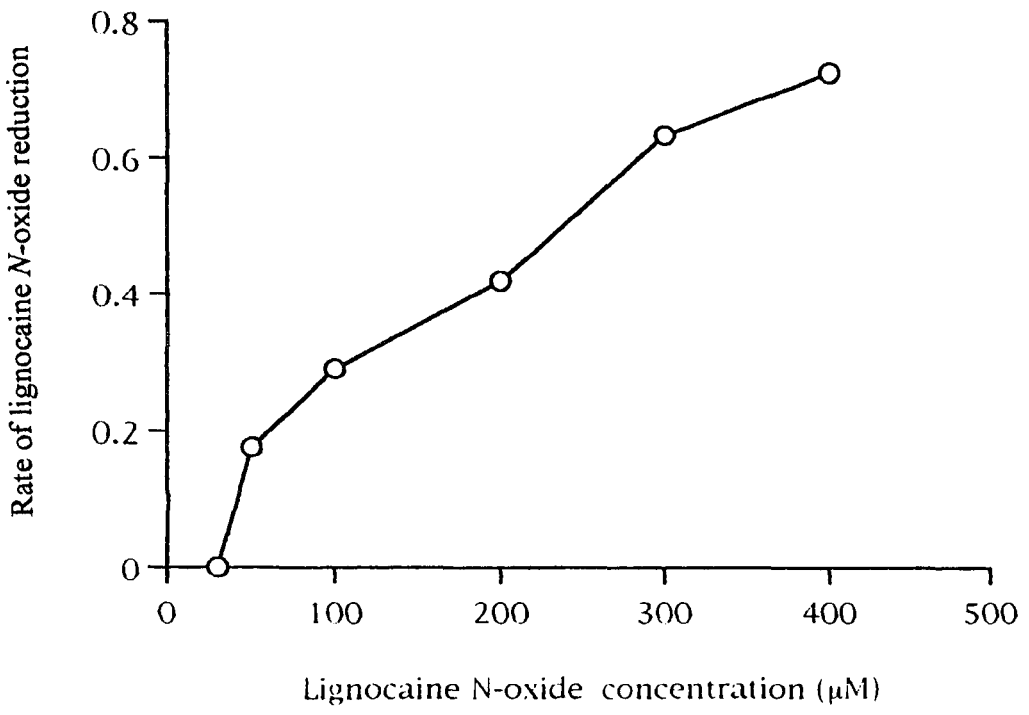


Figure 3-16 Effect of lignocaine *N*-oxide concentration on its reduction in NADPH supplemented rat heart *S9* fraction under anaerobic conditions. Experimental details were as described in section 2.3. Results are the mean ± s.d. of three determinations.

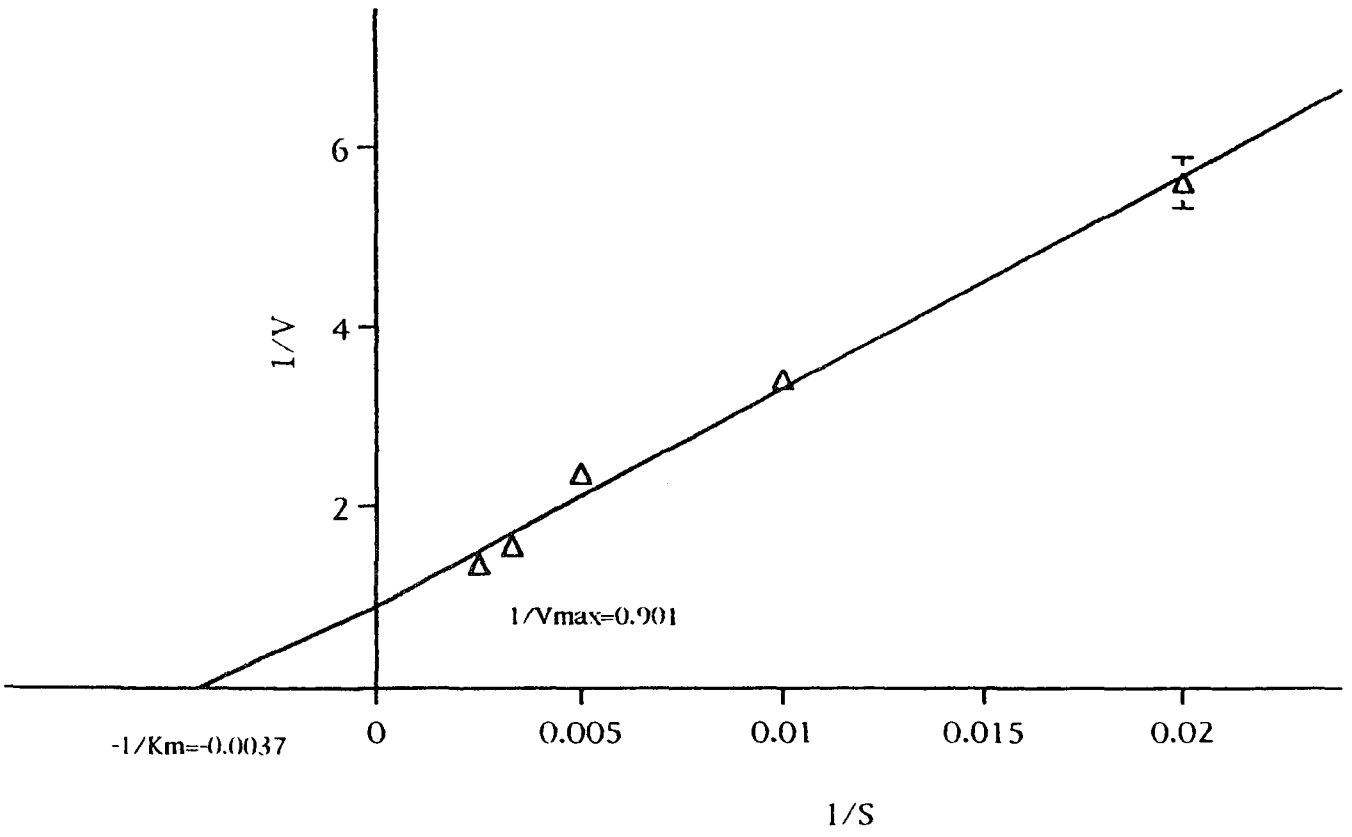


Figure 3-17 Lineweaver-Burk plot for lignocaine *N*-oxide reduction in NADPH supplemented rat heart *S9* fractions. Experimental details as described in section 2.3.

Linear regression equation : Y = 0.901 + 243.087 X
Correlation coefficient (r) = 0.990
Km = 269.8 μM
Vmax = 1.11 nmole/minute/mg protein

3.2.3 Lignocaine *N*-oxide metabolism in isolated perfused rat heart

Lignocaine *N*-oxide metabolism was investigated in a functional isolated heart preparation (Langendorff preparation). Carefully controlled conditions including temperature, oxygen and sodium-calcium balance perfusion fluid were applied to isolated rat hearts to achieve a steady (normal) cardiac rhythm. Anoxic/arrhythmic conditions was achieved by stopping the perfusion fluid.

Analysis of the homogenates of the perfused and non-perfused rat hearts showed that under anoxic condition, 46% of lignocaine *N*-oxide associated with the myocardium was metabolised to lignocaine; whilst no metabolism was found in the drug perfused rat hearts under oxygen perfusion (Table 3-4). Lignocaine was not found in the perfusion fluids collected from either normal (perfused) or anoxic (non-perfused) hearts. No lignocaine *N*-oxide was found in the perfusion fluid from the lignocaine perfused hearts under normal or anoxic conditions. A physiological difference was observed between control (Tyrode's solution), lignocaine and the lignocaine *N*-oxide perfused rat hearts under anoxic conditions. Normally an isolated perfused rat heart beats for only 1-2 min after stopping the oxygen supply, this was observed in the control and lignocaine perfused rat hearts, whereas the lignocaine *N*-oxide perfused hearts were found to beat for a further 6-8 min after stopping the perfusion of oxygen.

3.2.4 Lignocaine *N*-oxide metabolism following intraperitoneal administration to the rat

The fate of lignocaine *N*-oxide following *i.p.* administration to a healthy male rat was investigated. Urine samples (24 hr) were collected from the lignocaine *N*-oxide dosed rat (3.07 mg). The amount of lignocaine *N*-oxide and lignocaine collected in the urine

Table 3-4 Comparison of lignocaine *N*-oxide metabolism in normal and anoxic isolated perfused rat hearts.

Compound	Amount detected (n mole/ g of heart)	
	Perfused heart	Non-perfused heart
lignocaine N-oxide	129	71.0
lignocaine	0	61.5
lignocaine N-oxide Uptake*	129	132.5

Perfused (oxic) and non-perfused (anoxic) rat hearts were prepared as described in section 2.3.7.

*Following perfusion of lignocaine *N*-oxide (500 μM) to an isolated perfused heart (see section 2.3.7, the amount of drug uptake by the heart tissue was presumed to be the addition sum of lignocaine N-oxide plus lignocaine detected.

was 3.10 mg and 0.18 mg, respectively. This gave a recovery of lignocaine and lignocaine *N*-oxide of 100.9% and 5.7% respectively of the dosed lignocaine *N*-oxide. No interfering peaks were detected in the HPLC chromatograms. There was no detectable degradation of lignocaine *N*-oxide when it was stored at room temperature in rat urine for 24 hours. The total amount of lignocaine and lignocaine *N*-oxide recovered was over 100%. This cause of this deviation is unknown. Further investigation using more replicates (rats) and better control of sample handling may be required.

3.2.5 Lignocaine *N*-oxide metabolism in phenotyped human microsomes and in isolated rabbit myocytes

The metabolism of lignocaine *N*-oxide in a panel of human liver microsomes is shown in Table 3-5. Lignocaine *N*-oxide was selectively metabolised by several phenotyped human microsomes. According to the data analysis by Dr. Colin Henderson (ICRF, Dundee) using the Spearman correlation, no correlation was demonstrated between a specific P450 isoform and lignocaine *N*-oxide reduction.

No reduction was observed in the anaerobic incubation of lignocaine *N*-oxide with the isolated rabbit myocytes.

Table 3-5 Lignocaine *N*-oxide metabolism using phenotyped human liver microsomes

Human liver microsome	lignocaine N-oxide detected (%)	lignocaine detected (%)
IV	80.5	9.8
V	91.6	3.2
VII	61.0	31.0
VIII	81.4	26.5
IX	92.0	0.0
X	89.6	2.3
XI	97.3	0.0
XII	90.2	0.4
XIII	34.6	63.3
XIV	93.7	2.1
W1	59.5	38.4
B1	71.7	11.7

Experimental details were as described in section 2.3.8.

Each panel of phenotyped human liver microsomes containing different levels of cytochrome P450 isoforms. The human liver microsomes and data analysis came from Dr. Colin J. Henderson, Imperial Cancer Research Fund, Mol. Pharm. Unit, Biomedical Research Center, Ninewells Hospital & Medical School, Dundee.

3.3 Mechanistic studies of lignocaine *N*-oxide reduction

3.3.1 Effect of selected inhibitors on lignocaine *N*-Oxide metabolism in rat liver and heart tissue

The effect of various inhibitors on the reduction of lignocaine *N*-oxide was studied in order to verify that the reduction process was enzymically mediated. As shown in Table 3-6 carbon monoxide totally inhibited lignocaine *N*-oxide reduction in both NADPH supplemented rat liver microsomes and heart S9 fractions. Under aerobic conditions, lignocaine *N*-oxide reduction were decreased substantially in both rat liver microsomes and heart S9 fractions. In rat liver microsomes potassium cyanide treatment or denaturing the protein completely inhibited *N*-oxide reduction. However, in rat heart S9 fraction, these treatments diminished but did not abolish lignocaine reduction. When lignocaine (200 μM) was added to rat microsomes prior to measuring lignocaine *N*-oxide metabolism, the amount of *N*-oxide metabolised decreased from 63.5% to 33.5%.

3.3.2 Spectral studies of lignocaine *N*-oxide and lignocaine binding to rat liver microsomes

Rat liver microsomes in a reduced and an oxidised state were used to examine the binding between substrate (lignocaine *N*-oxide or lignocaine) and microsomal cytochrome P450.

Spectral changes induced by lignocaine *N*-oxide and lignocaine binding to the oxidised and reduced rat liver microsomes were recorded between 350 nm and 500 nm.

Table 3-6 Effect of various treatments on lignocaine *N*-oxide reduction in NADPH supplemented rat liver microsomes and rat heart S9 fraction.

Inhibitors	Lignocaine N-oxide reduction (%)	
	liver microsomes	heart s9 fraction
Nitrogen	63.5±2.0	87.2±1.7
Air	15.3±2.4	22.7±2.1
Carbon monoxide	0	0
Heat denatured tissue	0	47.4±3.5
KCN (100μM)	0	33.5±1.3
Lignocaine (200μM)	33.8±1.1	(Not determined)

All procedures were carried out following experimental details as described in section 2.4.1. All results are the mean ± standard deviation of three replicates.

In the oxidised microsomes, lignocaine *N*-oxide and lignocaine produced similar difference spectra with a trough at 417 nm as shown in Figure 3-18 and Figure 3-19. Figure 3-20 and Figure 3-21 show the difference spectra recorded from the addition of lignocaine *N*-oxide and lignocaine to the reduced rat liver microsomes. Spectral changes with the maximum absorbance at 410 nm and at 426 nm were shown for both lignocaine and lignocaine *N*-oxide.

The spectral changes of lignocaine *N*-oxide and lignocaine in either oxidised or reduced microsomes are consistent with the typical spectrum of Type I binding of cytochrome P450.[193]

From Figure 3-22 A and Figure 3-23A, the magnitude of the substrate-oxidised microsomes binding spectra has been shown to be proportional to the concentrations of lignocaine *N*-oxide or lignocaine. The spectral dissociation constants (Ks) of lignocaine and lignocaine *N*-oxide binding to either oxidised or reduced microsomes were determined using the Lineweaver-Burke linear transformation. From the data of lignocaine and lignocaine *N*-oxide binding to the oxidised microsomes (Figure 3-22B), the values for Ks were determined to be 137.13 μM and 250.34 μM, respectively. From the data of lignocaine and lignocaine *N*-oxide binding to the reduced microsomes (Figure 3-23B), the values for Ks were determined to be 21.54 μM and 1.45 μM, respectively.

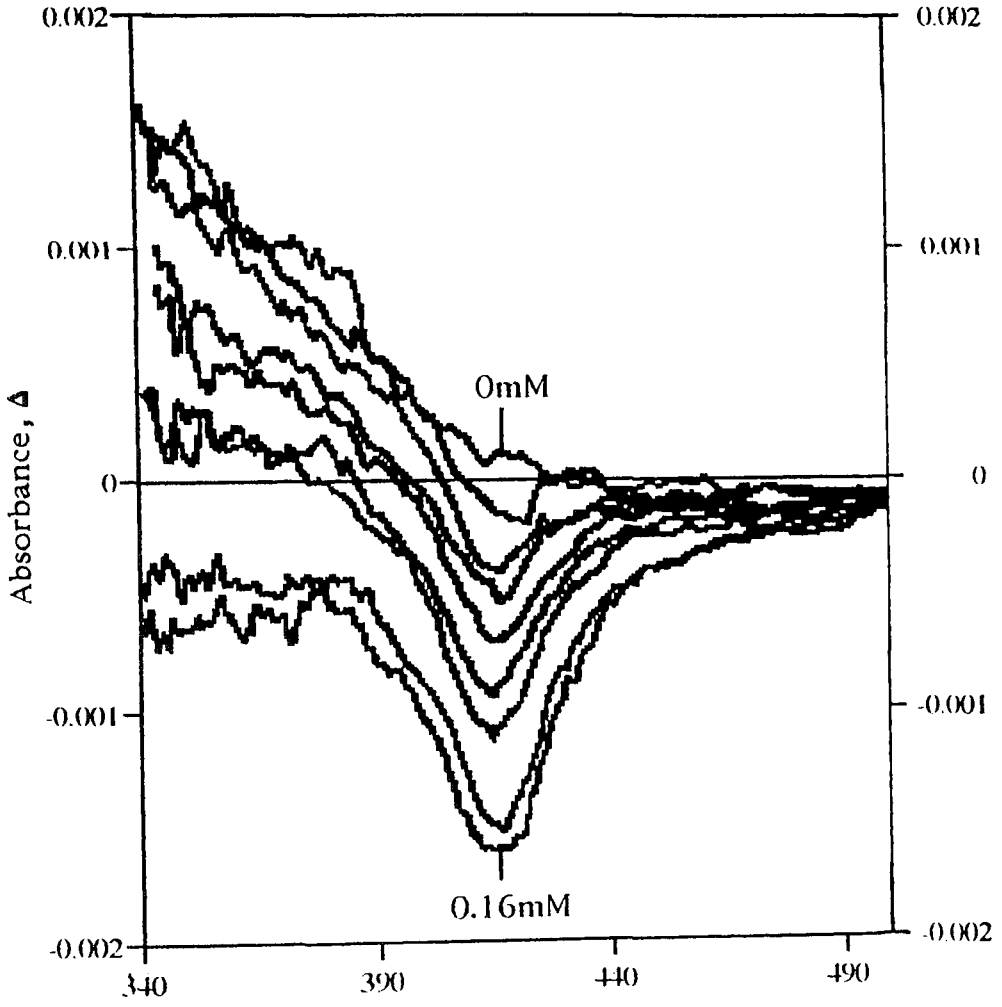


Figure 3-18 Visible difference spectra of lignocaine *N*-oxide and oxidised rat liver microsomal suspensions. Lignocaine *N*-oxide concentrations were 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14 and 0.16 mM. Experimental details were as described in section 2.4.2.

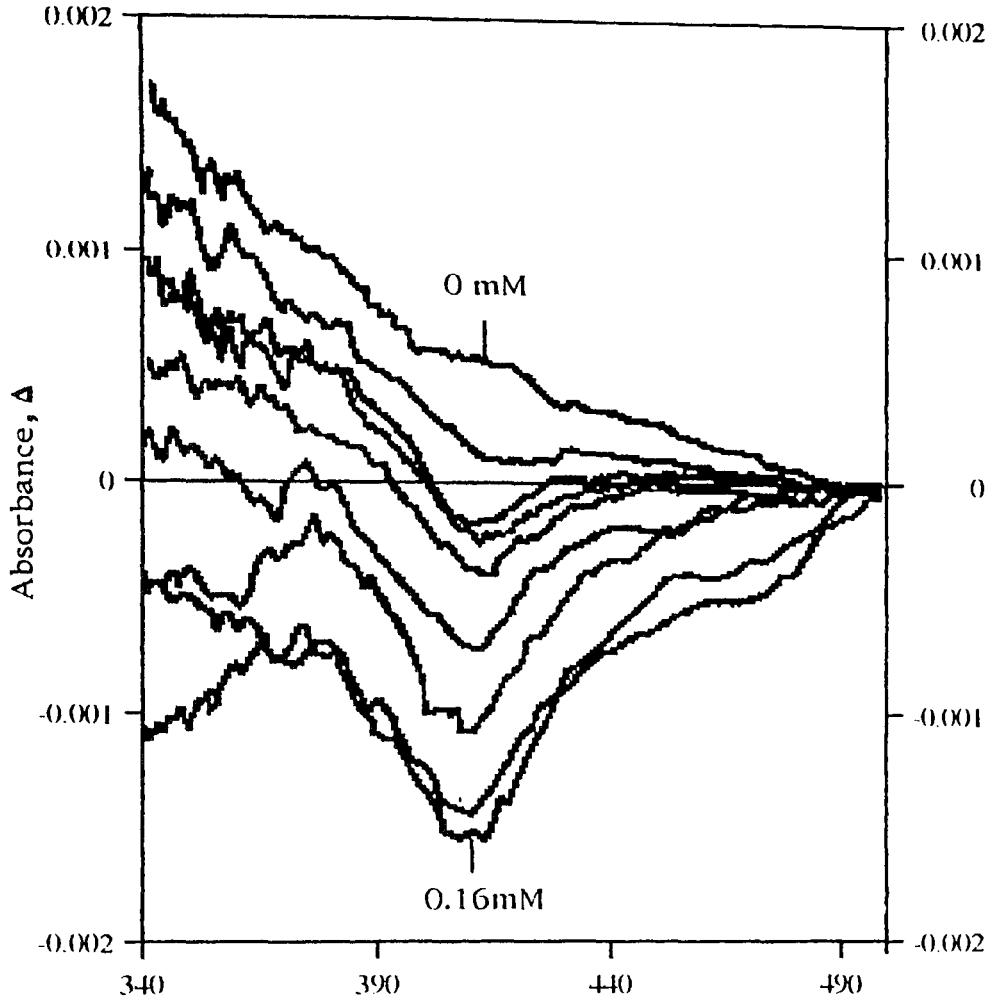


Figure 3-19 Visible difference spectra of lignocaine and oxidised rat liver microsomal suspensions. Lignocaine concentrations were 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.14 and 0.16 mM. Experimental details were as described in section 2.4.2.

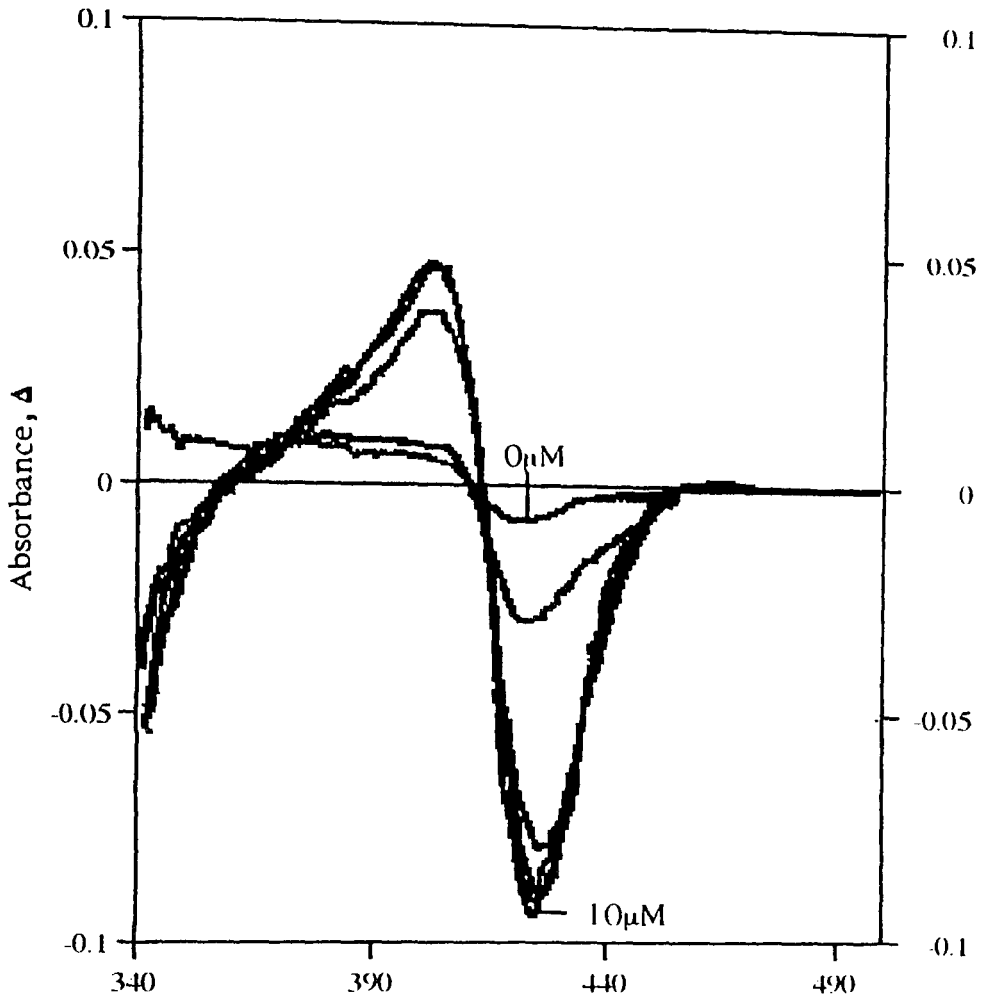


Figure 3-20 Visible difference spectra of lignocaine *N*-oxide and reduced rat liver microsomal suspensions. Lignocaine *N*-oxide concentrations were 0, 2, 4, 6, 8 and 10 μM. Experimental details were as described in section 2.4.2.

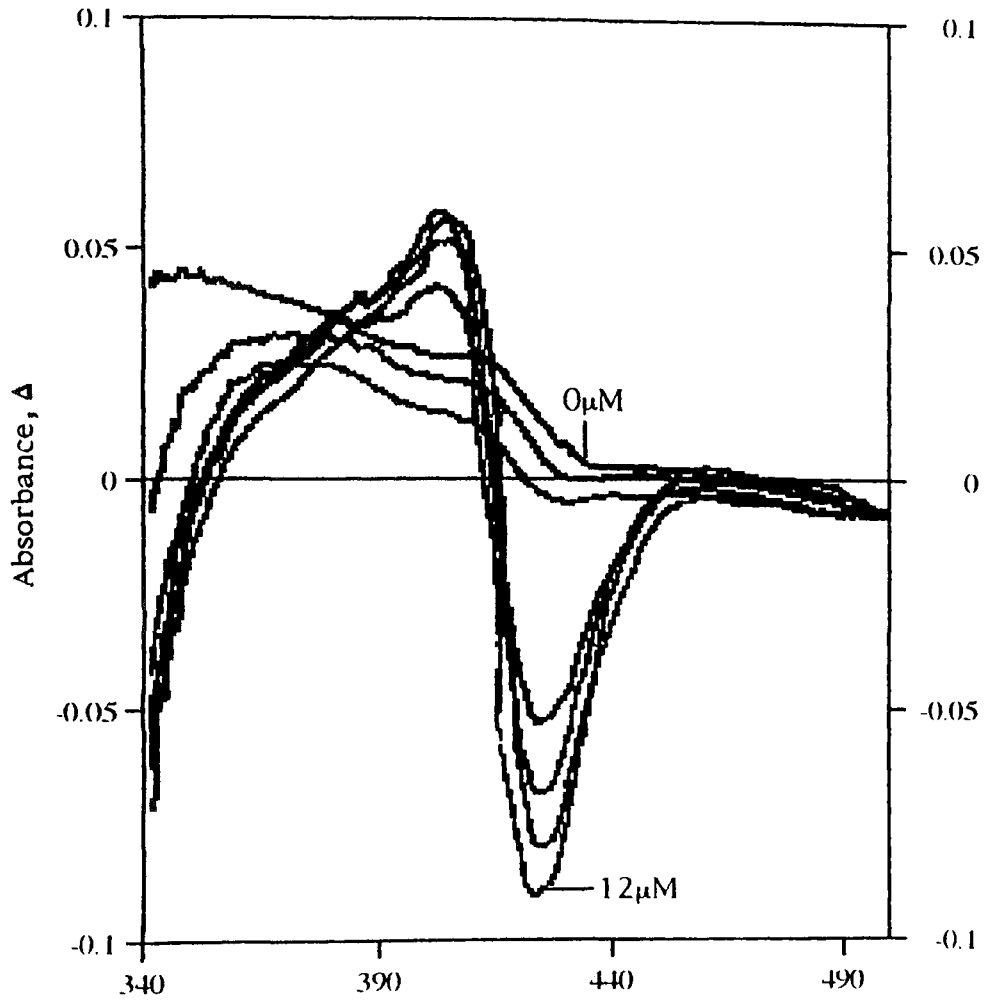


Figure 3-21 Visible difference spectra of lignocaine and reduced rat liver microsomal suspensions. Lignocaine concentrations were 0, 2, 4, 6, 8, 10 and 12 μM. Experimental details were as described in section 2.4.2.

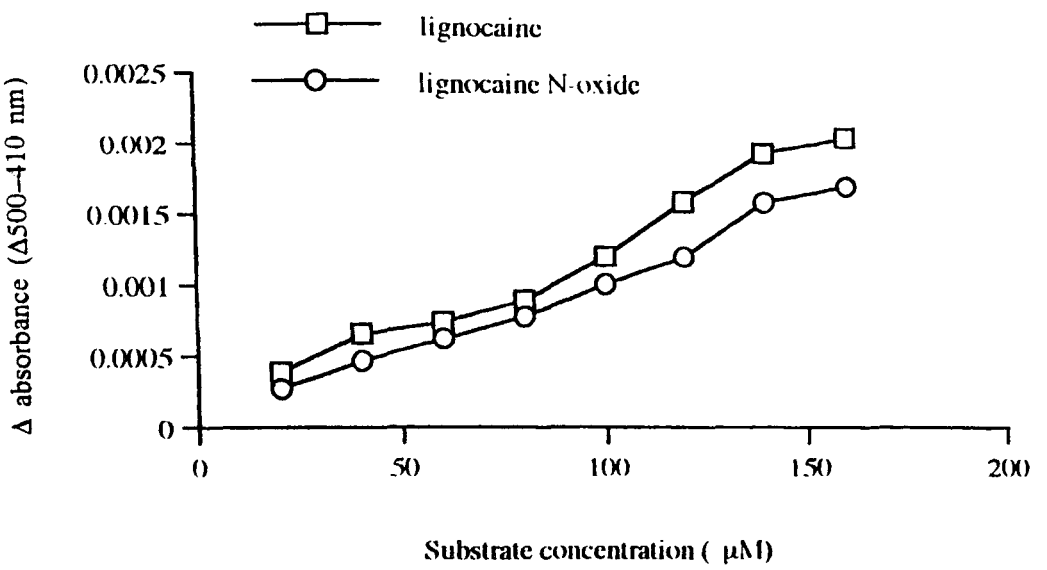


Figure 3-22 (A) Effect of lignocaine *N*-oxide and lignocaine concentration on the magnitude of the difference spectrum (△500-410nm) in oxidised rat liver microsomes. Experimental details were as described in section 2.4.2.

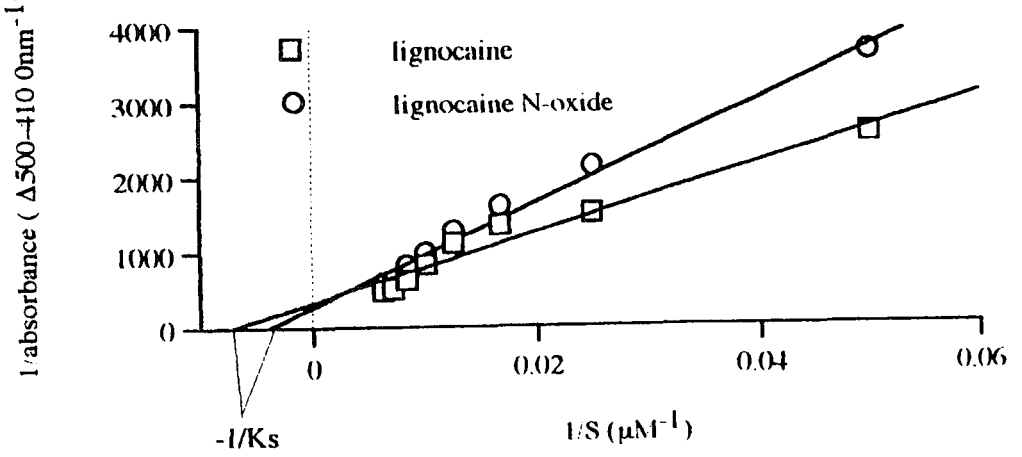


Figure 3-22 (B) Lineweaver-Burk plot of lignocaine *N*-oxide and lignocaine concentrations against the magnitudes of the difference spectrum in oxidised rat liver microsomes. Experimental details were as described in section 2.4.2.

Linear regression equation :	
lignocaine	Y = 46503.206X +339.111
lignocaine N-oxide	Y = 70100.561X + 280.026
Correlation coefficient (r^2):	
lignocaine	0.953
lignocaine N-oxide	0.985
Spectral dissociation constant (Ks):	
lignocaine	137.13 μM
lignocaine N-oxide	250.34 μM

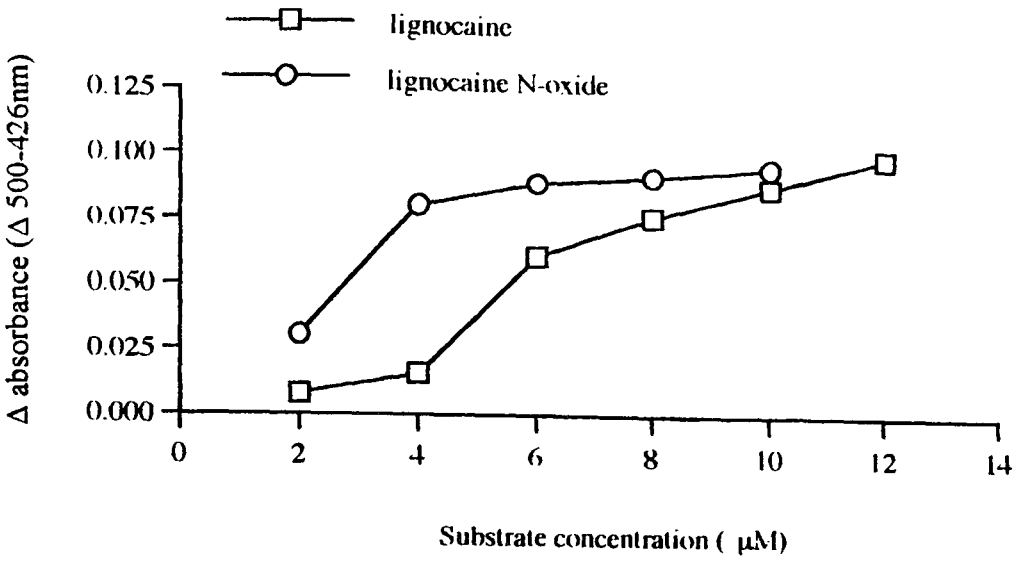


Figure 3-23 (A) Effect of lignocaine *N*-oxide and lignocaine concentration on the magnitude of the difference spectrum (△500nm-410nm) in reduced rat liver microsomes. Experimental details were as described in section 2.4.2.

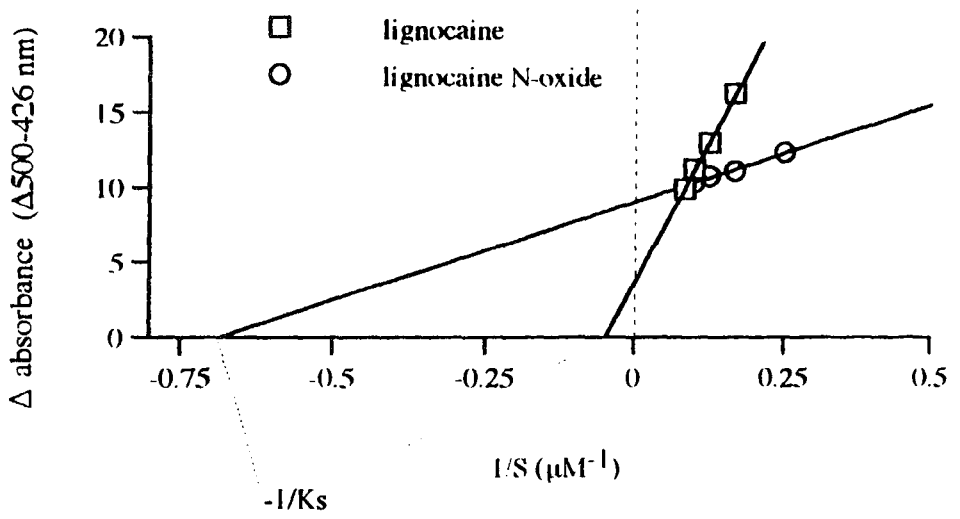


Figure 3-23 (B) Lineweaver-Burk plot of lignocaine *N*-oxide and lignocaine concentrations against the magnitudes of the difference spectrum in reduced rat liver microsomes. Experimental details were as described in section 2.4.2.

Linear regression equation :

lignocaine	Y = 76.979X + 3.574
lignocaine N-oxide	Y = 13.206X + 9.108

Correlation coefficient (r^2):

lignocaine	0.999
lignocaine N-oxide	0.991

Spectral dissociation constant (Ks):

lignocaine	21.54 μM
lignocaine N-oxide	1.45 μM

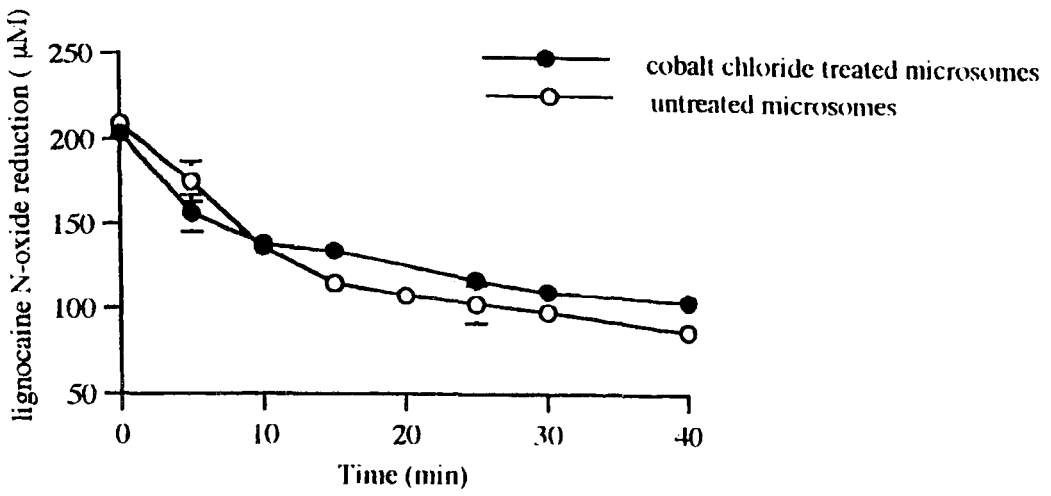
3.3.3 Lignocaine *N*-oxide reduction in microsomes prepared from cobalt dosed rats

The amounts of cytochrome P450 and b_5 in control and cobalt treated rat liver microsomes were measured. Administration of cobalt decreased the amount of both cytochrome P450 and b_5, from 2.2 to 0.9 and 3.67 to 2.25 (n mole /mg protein), respectively, however lignocaine *N*-oxide was metabolically reduced in liver microsomes prepared from both control and cobalt treated rats. After an anaerobic incubation of 40 min, lignocaine *N*-oxide reduction in control rat liver microsomes was 63%; in the cobalt treated rat liver microsomes it was 45% (Figure 3-24). Therefore metabolism of lignocaine *N*-oxide was diminished as the content of cytochrome P450 and b_5 decreased.

3.3.4 Reduction of lignocaine *N*-oxide in the presence of P450 reductase, haem oxygenase or xanthine oxidase

Lignocaine *N*-oxide is quantitatively converted into lignocaine when incubated under anaerobic incubation for 60 min in the presence of cytochrome P450 reductase, haemin and NADPH (Table 3-7). Incubation in the absence of cytochrome P450 reductase, NADPH or haem resulted in no lignocaine *N*-oxide reduction. Lignocaine *N*-oxide was fully reduced by xanthine oxidase with or without the supplement of haem. The reduction of lignocaine *N*-oxide was shown to be independent of human recombinant haem oxygenase.

(A)



(B)

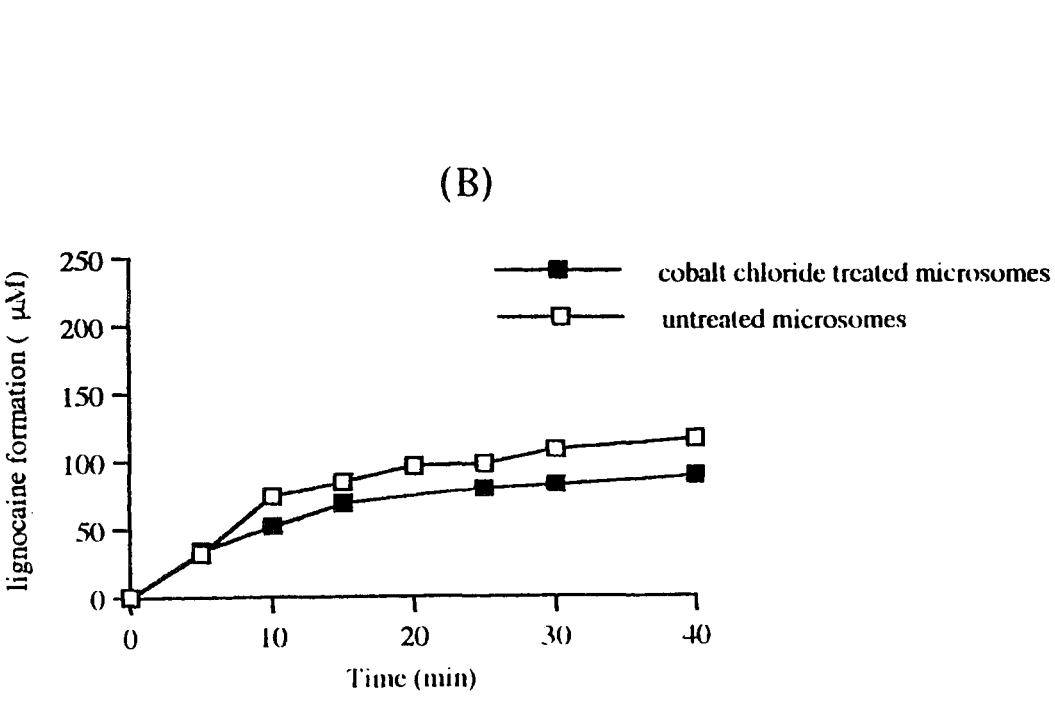


Figure 3-24 Anaerobic incubation of lignocaine *N*-oxide in NADPH supplemented cobalt chloride treated and untreated rat liver microsomes: (A) reduction of lignocaine *N*-oxide (B) formation of lignocaine. Experimental details were as described in section 2.4.3. Results are the mean ± s.d. of three determinations.

Table 3-7 Effect of haem, NADPH, haem oxygenase and cytochrome P450 reductase on lignocaine *N*-oxide metabolism

Component added	Incubation mixtures						
	1	2	3	4	5	6	7
lignocaine *N*-oxide (100 μM)	√	√	√	√	√	√	√
cytochrome P450 reductase (3 nmole)	–	√	√	√	√	–	–
Haemoxygenase (0.7 nmole)	√	–	√	√	√	–	–
haem (40 μM)	√	√	√	–	√	√	–
NADPH (200 μM)	√	√	√	√	–	√	√
xanthine oxidase(1 unit)	–	–	–	–	–	√	√
Results: Percentage of lignocaine *N*-oxide reduction after 1hr of anaerobic incubation.	none	100%	100%	none	none	100%	100%

All procedures were carried out following experimental details as described in section 2.4.4. All results are the mean ± standard deviation of three replicates.

"√" Indicates the components present in the total incubation mixtures.

3.4 Non-enzymic reduction of lignocaine *N*-Oxide

3.4.1 Lignocaine *N*-oxide reduction by myoglobin and haemoglobin

Lignocaine *N*-oxide was anaerobically incubated with deoxymyoglobin and metmyoglobin to investigate the effect of myoglobin oxidative state on lignocaine *N*-oxide reduction. Lignocaine *N*-oxide was 46% metabolised to lignocaine in the presence of deoxymyoglobin after an anaerobic incubation of 2 hr (Figure 3-25). No reduction was detected in the presence of metmyoglobin. Lignocaine *N*-oxide reduction by deoxymyoglobin solution was demonstrated to be inhibited by oxygen (Figure 3-26). As the oxygen content increased from 0 to 3.5%, the amount of lignocaine *N*-oxide reduction decreased from 44.8% to 6.6% following a 2 hour incubation; lignocaine *N*-oxide reduction being completely inhibited when oxygen concentration was 5% or greater.

No reduction was observed during the anaerobic incubation of lignocaine *N*-oxide with either deoxyhaemoglobin or methaemoglobin with the supplement of NADPH.

3.4.2 Lignocaine *N*-oxide reduction by inorganic iron and haem

From Table 3-8 it can be seen that lignocaine *N*-oxide was quantitatively converted into lignocaine by haemin in the presence of ascorbic acid; whilst only a minor amount (5%) of lignocaine *N*-oxide was reduced by haemin-NADPH combination. No reduction occurred in the presence of either Fe^{2+}, Fe^{3+}, haemin alone or NADPH alone.

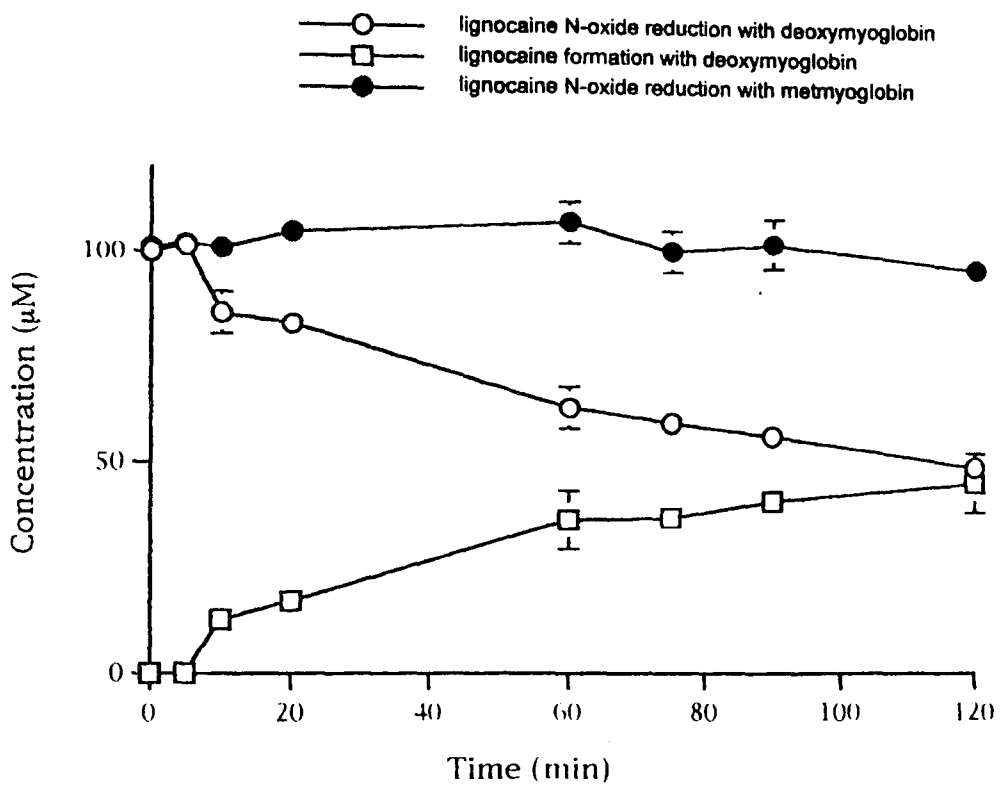


Figure 3-25 Anaerobic incubation of lignocaine *N*-oxide with deoxymyoglobin and metmyoglobin. Experimental details as described in section 2.4.5. Results are the mean ± s.d. of three determinations.

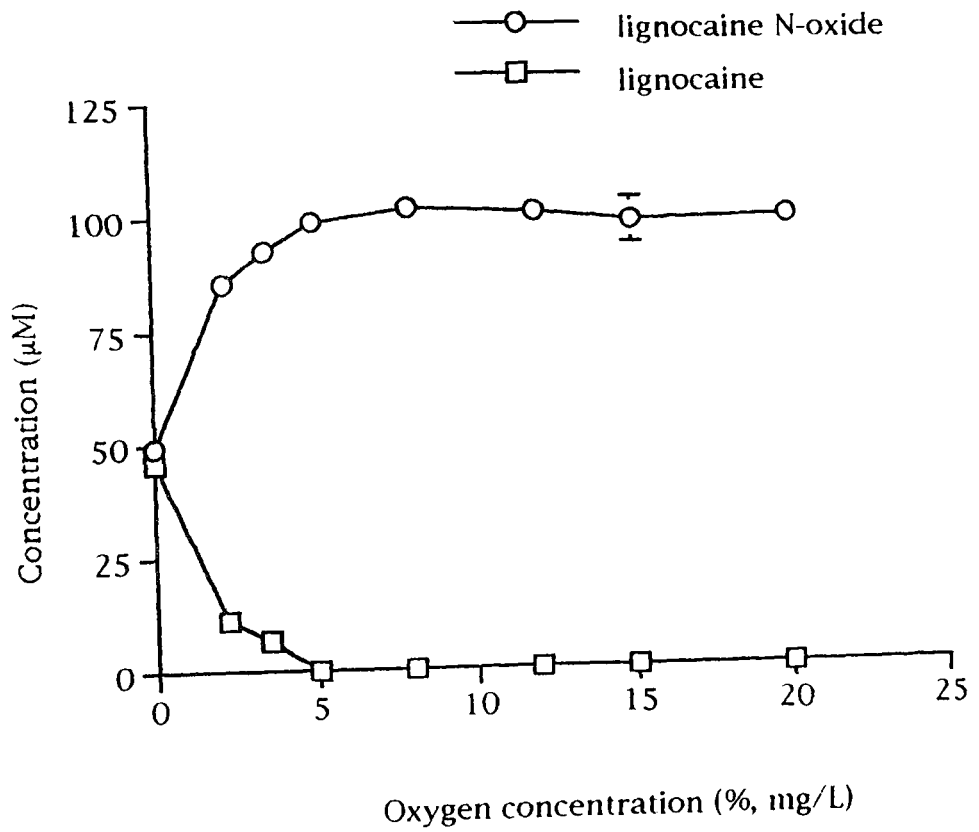


Figure 3-26 Effect of oxygen tension on the reduction of lignocaine *N*-oxide by deoxymyoglobin. Experimental details as described in section 2.4.5. Results are the mean ± s.d. of three determinations.

Table 3–8 Effect of NADPH, iron, haemin and ascorbic acid on lignocaine *N*-oxide metabolism.

Component added	Incubation mixtures									
lignocaine N-oxide (100 μM)	√	√	√	√	√	√	√	√	√	√
Fe^{2+} (1 mM)	√	√								
Fe^{3+} (1 mM)			√	√						
EDTA (1 mM)		√		√		√				
haemin (1 mM)					√	√	√	√		
ascorbic acid (1 mM)							√		√	
NADPH (1 mM)								√		√
lignocaine N-oxide reduction (%)	0	0	0	0	0	0	100	5	0	0

All procedures were carried out following experimental details as described in section 2.4.7. All results are the mean ± standard deviation of three replicates. NADPH was prepared from NADPH generating system as described in section 2.2.2.

"√" indicates the components present in the total incubation mixtures.

3.4.3 Electron spin resonance spectroscopy studies of lignocaine *N*-oxide in the presence of myoglobin

ESR spectroscopy in combination with the spin trapping agent, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), was used to investigate further the mechanism of lignocaine *N*-oxide reduction mediated by myoglobin.

In order to assist interpretation of the spin trap data from lignocaine *N*-oxide reduction by myoglobin, model systems were first used to identify possible DMPO radical species. With a suitable oxidising system (see Figure 3-27), the hydroxyl radical ($OH^{\bullet}$) DMPO/$OH^{\bullet}$ (1:2:2:1 spectrum with hyperfine splitting constants, $A_N = A_H$ =14.9 G) and the carbon centred alkyl radical ($CH_3^{\bullet}$ or $R^{\bullet}$) DMPO/ $R^{\bullet}$ (1:1:1:1:1:1) ESR spectrum with hyperfine splitting constants, A_N =16.2 G, A_H =23.0 G) spin adducts were observed (See Figure 3-27 A and B). These results were consistent with previous studies using DMPO to trap hydroxyl and alkyl free radicals.[194] Further evidence that these spectra were derived from reactive oxygen is shown by loss of the DMPO/$OH^{\bullet}$ ESR spectrum when superoxide dismutase (SOD) and catalase were added (Figure 3-27 C). SOD and catalase will compete with DMPO for reactive oxygen.[3]

In the anaerobic incubation of lignocaine *N*-oxide and deoxymyoglobin, a DMPOX (5,5-dimethylpyrrolidone-2-oxy-1) and DMPO/$R^{\bullet}$ ESR spectrum were both observed (see Figure 3-28 B).

MbFe(II)+ DMPO + lignocaine N-oxide →DMPO/R• + DMPOX + MbFe(III) (final product)

The DMPOX ESR signal was shown to decrease with time, whilst the DMPO/ $R^{\bullet}$ spectrum appeared to persist over a 10 minute period. Under anaerobic conditions, no spin trapped ESR spectrum was detected from deoxymyoglobin alone (Figure 3-28 A), lignocaine and deoxymyoglobin (Figure 3-28 D) or metmyoglobin and lignocaine *N*-oxide (Figure 3-28 G). Also, no ESR signal was observed in the presence of deoxymyoglobin and lignocaine *N*-oxide or lignocaine under aerobic conditions

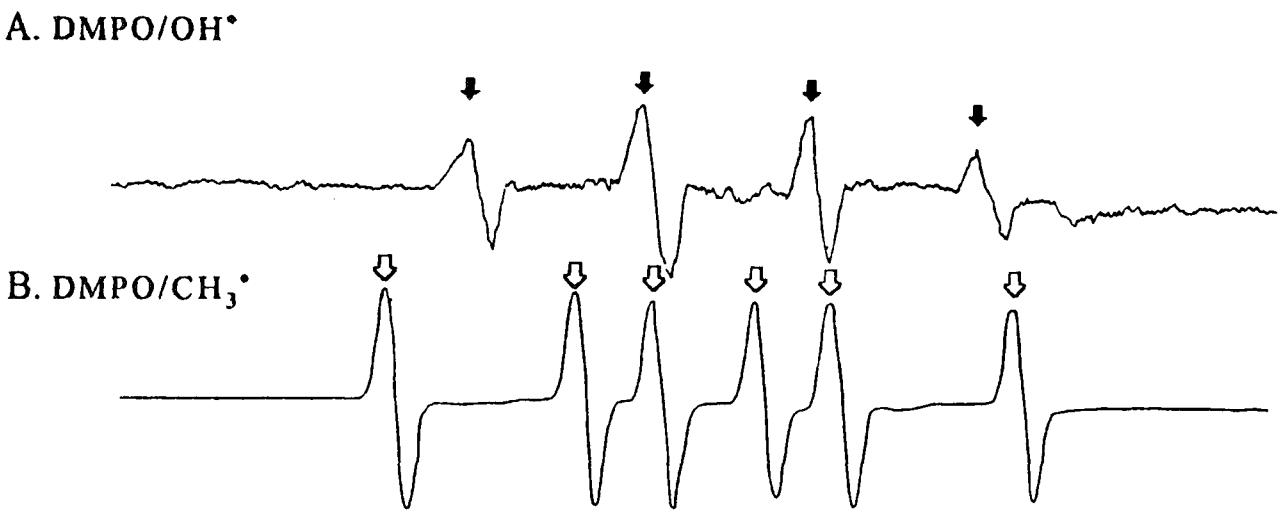


Figure 3-27 The 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin adduct electron spin resonance spectra of:

A. DMPO/OH• ⬇ Fe^{2+} + EDTA + H_2O_2 + DMPO

B. DMPO/CH_3• or DMPO/R• ⇩ Fe^{2+} + EDTA + H_2O_2 +DMSO + DMPO

C. $FeSO_4$ + EDTA + H_2O_2 + DMPO + superoxide dismutase + catalase

Experimental details were as described in section 2.4.8.

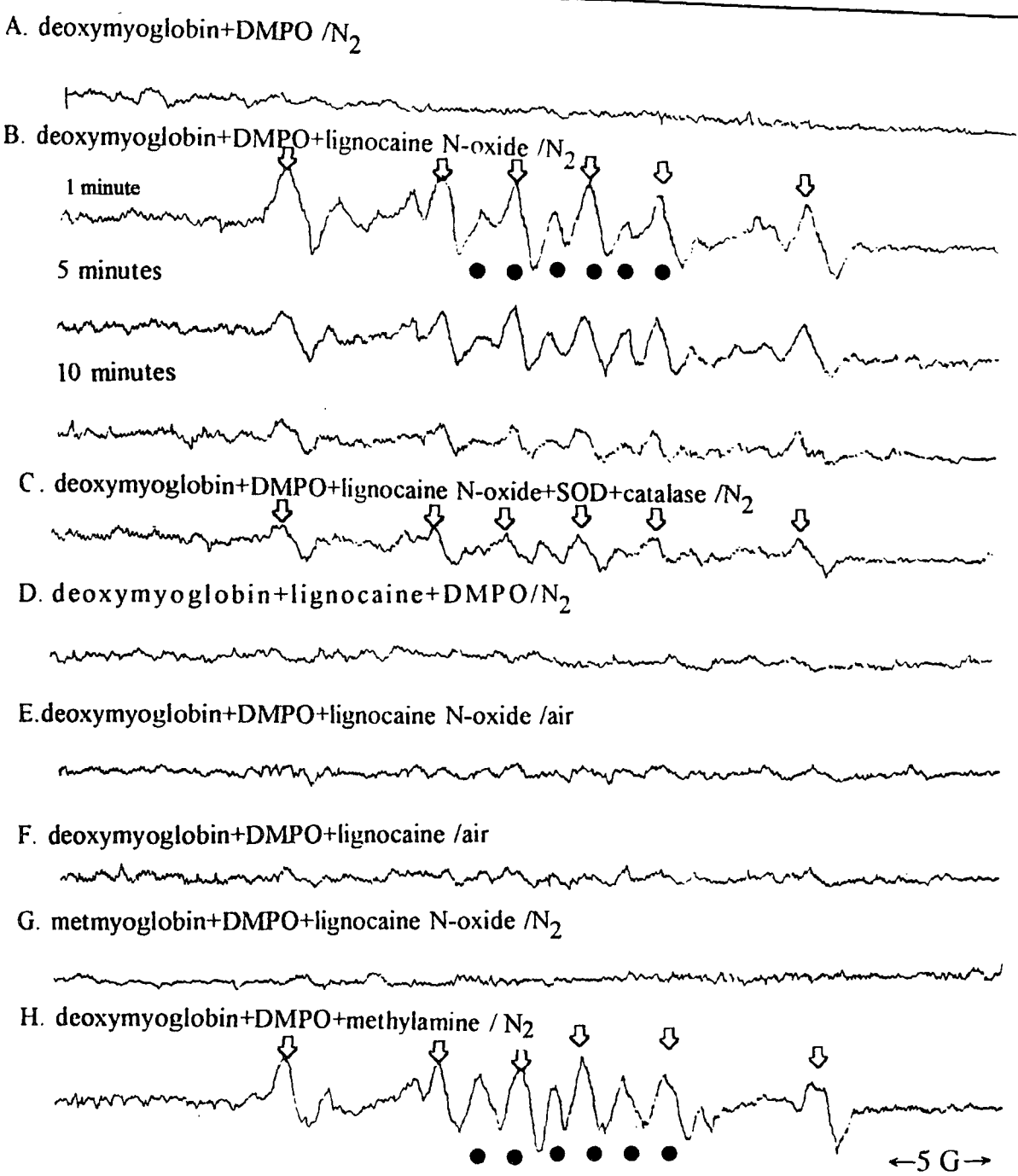


Figure 3-28 The 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin adduct electron spin resonance spectra of:

A. deoxymyoglobin+DMPO

B. deoxymyoglobin+DMPO+lignocaine N-oxide anaerobically incubated at 37 °C at 0, at 1, 5 and 10 minutes DMPOX+DMPO/R•

C. deoxymyoglobin+lignocaine N-oxide +SOD+catalase+DMPO DMPO/R•

D. deoxymyoglobin + DMPO + lignocaine

E. deoxymyoglobin +lignocaine N-oxide+DMPO (air)

F. deoxymyoglobin +lignocaine +DMPO (air)

G. metmyoglobin+DMPO+lignocaine N-oxide

H. deoxymyoglobin+methylamine+DMPO. DMPOX+DMPO/R•

All experiments were operated under anaerobic conditions except E and F. ESR condition and experimental details were as described in section 2.4.8.

⇩ DMPO/R•

(Figure 3-28 E and F). However, a similar mixture of ESR spectra (DMPOX and DMPO/ $R^{\bullet}$) was observed with methylamine and deoxymyoglobin under anaerobic condition (Figure 3-28 H). The significance of this will be discussed later.

The ESR spectrum of DMPOX was identified previously and is the result of direct oxidation of DMPO.[195]

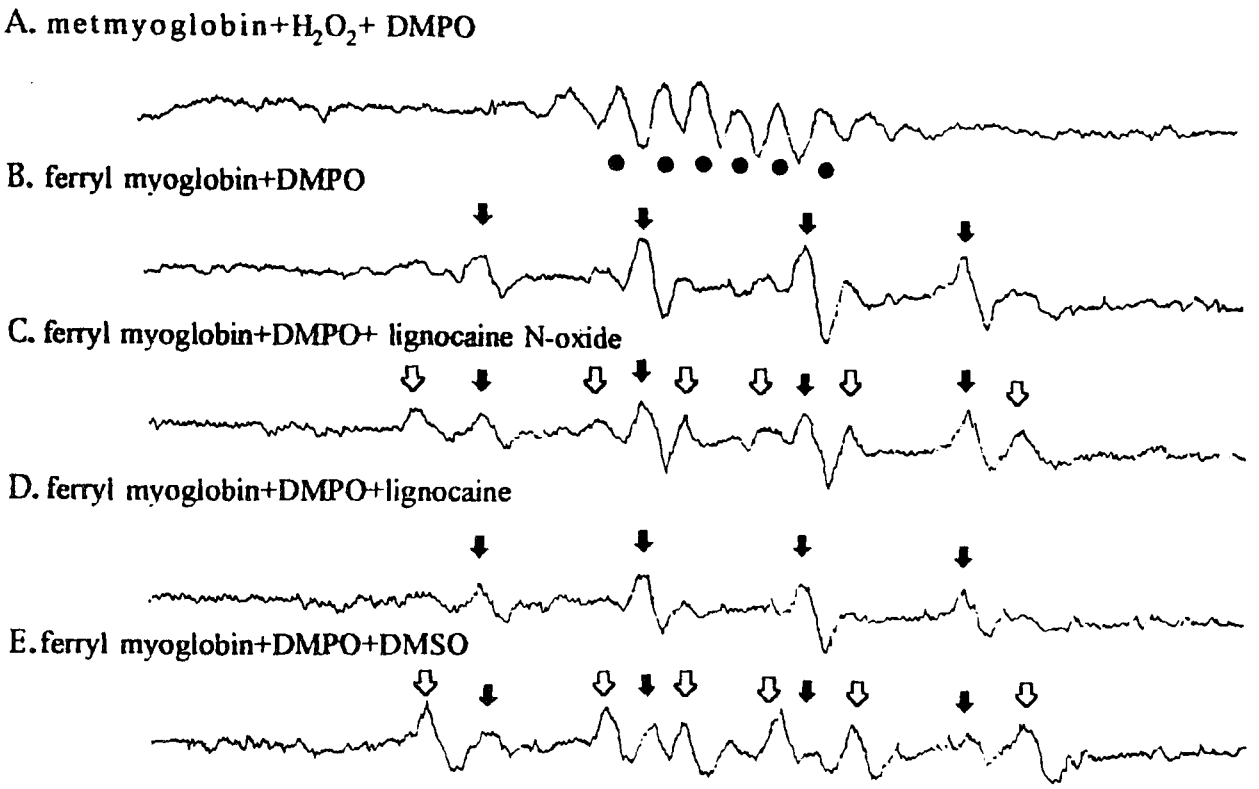
MbFe(III) + DMPO + Hydrogen peroxide → DMPOX + MbFe(IV)=O

Further evidence for a myoglobin mediated oxidation of DMPO to the DMPOX species was obtained using oxidation of metmyoglobin with hydrogen peroxide. This resulted in formation of a characteristic DMPOX ESR spectrum consisting of a triplet of doublets with hyperfine coupling constants A_N =7 G, A_H =4 G (see Figure 3-29 A).

A characteristic DMPO/$OH^{\bullet}$ spin adduct was detected in the presence of ferryl myoglobin [Fe(IV)=O] (prepared free from metmyoglobin as described in section 2.1.4) with or without the addition of lignocaine (See Figure 3-29 B and D). When lignocaine *N*-oxide or dimethylsulphoxide (DMSO) was added to the ferryl myoglobin and DMPO, a mixed spectrum of DMPO/$R^{\bullet}$ and DMPO/$OH^{\bullet}$ spin adducts was recorded (Figure 3-29 C and E).

Fe(IV)=O + DMPO + lignocaine N-oxide (or DMSO) → DMPO/$R^{\bullet}$ + DMPO/$OH^{\bullet}$ + MbFe(III)

The DMPO/OH spectrum is likely to be a direct result of DMPO trapping a hydroxyl radical liberated as ferryl myoglobin is oxidised to metmyoglobin. The ferryl myoglobin or its liberated hydroxyl radical will also react with a suitable hydrogen donor such as DMSO (see Figure 3-29 E) to liberate an alkyl radical that is trapped by DMPO as DMPO/ $OH^{\bullet}$. Lignocaine-*N*-oxide appears to react in a similar way with ferryl myoglobin (see Figure 3-29 C) since both lignocaine-*N*-oxide and DMSO result in DMPO/$R^{\bullet}$ adducts. The implications of this will be discussed later (section 4.3).



←5 G→

Figure 3-29 The 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin adduct electron spin resonance spectra of:

A. DMPOX	metmyoglobin+H_2O_2+ DMPO
B. DMPO/OH•	ferryl myoglobin+DMPO;
C. DMPO/OH•+DMPO/R•	ferryl myoglobin+DMPO+ lignocaine N-oxide
D. DMPO/OH•	ferryl myoglobin+DMPO+lignocaine
E. DMPO/OH•+DMPO/R•	ferryl myoglobin+DMPO+DMSO.

All experiments were operated under anaerobic condition. ESR condition and experimental details were as described in section 2.4.8.

●DMPOX

⇩ DMPO/R•

⬇ DMPO/OH•

3.4.4 Spectral binding studies of lignocaine *N*-oxide with myoglobin

Myoglobin has a characteristic absorbance spectrum in the visible region in the absence of oxygen. Spectral changes were observed for deoxymyoglobin and metmyoglobin in the presence of lignocaine *N*-oxide and lignocaine. As seen in Figure 3-30 and 3-31, the binding of either agents to myoglobin produced a difference spectra in wavelength from 250 to 440 nm, while the spectrum between 440 nm to 600 nm remained unchanged. The binding of lignocaine *N*-oxide and lignocaine to deoxymyoglobin produced a hypsochromic shift at 418 nm; a hypochromic shift for lignocaine and a hyperchromatic shift for lignocaine *N*-oxide at 355 nm (Figure 3-30). As shown in Figure 3-31, the binding of metmyoglobin to lignocaine *N*-oxide produced a hyperchromic shift, whilst the binding to lignocaine produced a hypochromic shift at 410 nm. The binding of ferryl myoglobin to lignocaine *N*-oxide and lignocaine both produced hyperchromic shifts in the absorbance at 288 nm as shown in Figure 3-32. These changes of absorption intensity indicate the binding between lignocaine and lignocaine *N*-oxide with myoglobin.

The UV/visible difference spectrum observed following an anaerobic incubation of lignocaine *N*-oxide with deoxymyoglobin at 37 ° C for 60 min along with the lignocaine *N*-oxide and deoxymyoglobin binding spectra is shown in Figure 3-33. Incubation of lignocaine *N*-oxide with deoxymyoglobin results in a diminution of the myoglobin spectral peaks at 544 and 582 nm with a concomitant increase at 418 nm.

Figure 3-34 shows the changes in absorbance at 580 nm that occurred when several tertiary amine *N*-oxides were anaerobically incubated with deoxymyoglobin for 2 hr. Lignocaine *N*-oxide, amiodarone *N*-oxide, diltiazem *N*-oxide and disopyramide *N*-oxide all caused a similar decrease in the absorbance at 580 nm.

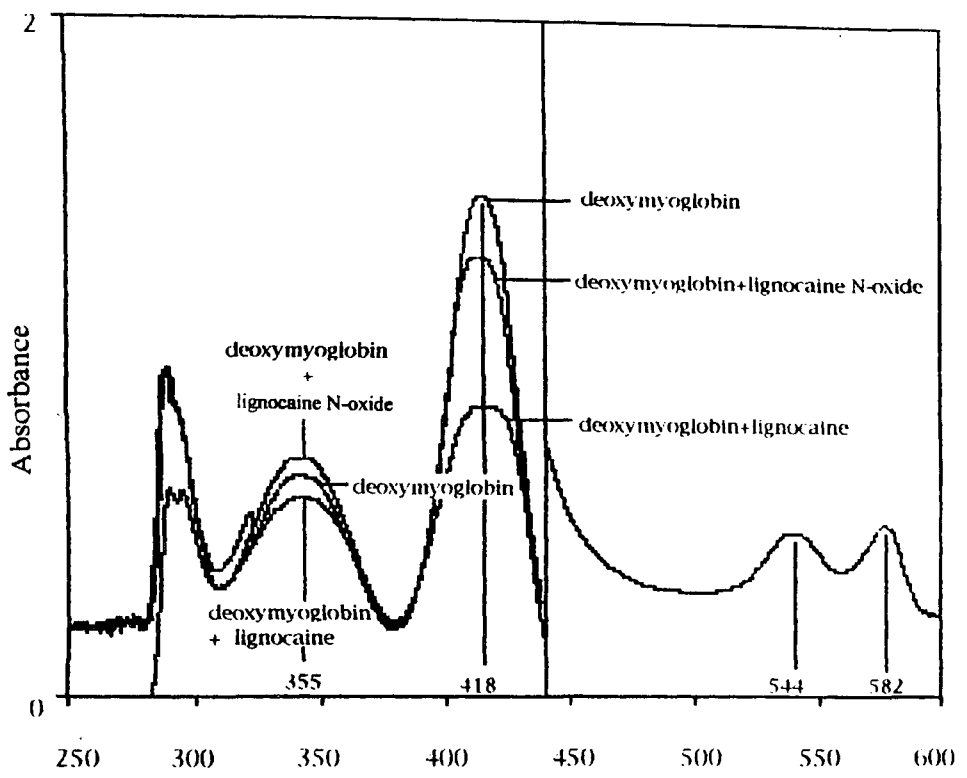


Figure 3-30 UV/visible difference spectra for deoxymyoglobin binding with lignocaine *N*-oxide and lignocaine. Experimental details were as described in section 2.4.9

*No spectral difference was observed in the deoxymyoglobin spectrum between 440 to 660 nm in the presence of lignocaine or lignocaine *N*-oxide.

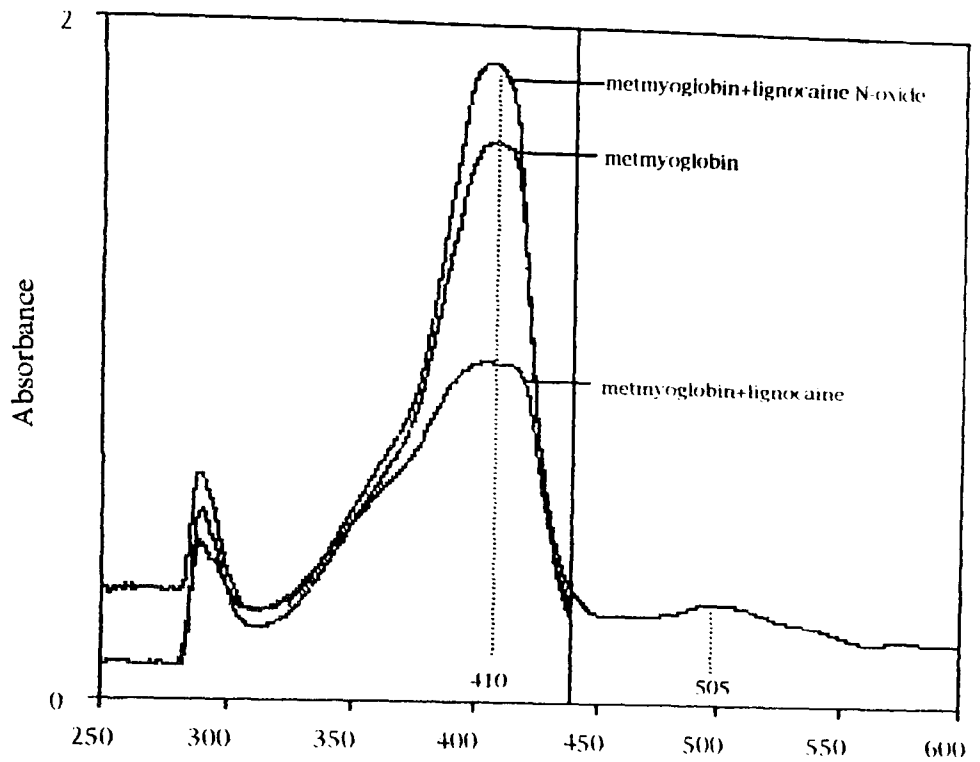


Figure 3-31 UV/visible difference spectra for metmyoglobin and metmyoglobin binding with lignocaine *N*-oxide and lignocaine. Experimental details were as described in section 2.4.9.

*No spectral difference was observed in the metmyoglobin spectrum between 440 to 660 nm in the presence of lignocaine or lignocaine *N*-oxide.

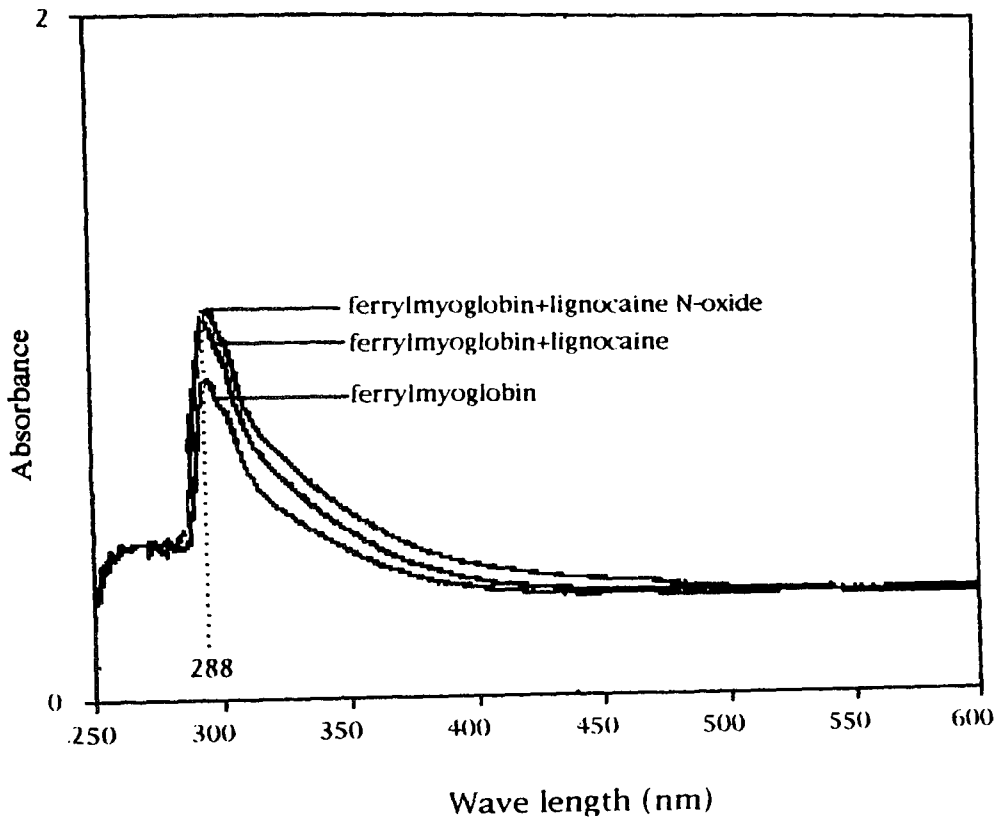


Figure 3-32 UV/visible difference spectrum for ferryl myoglobin binding with lignocaine *N*-oxide or lignocaine. Experimental details were as described in section 2.4.9.

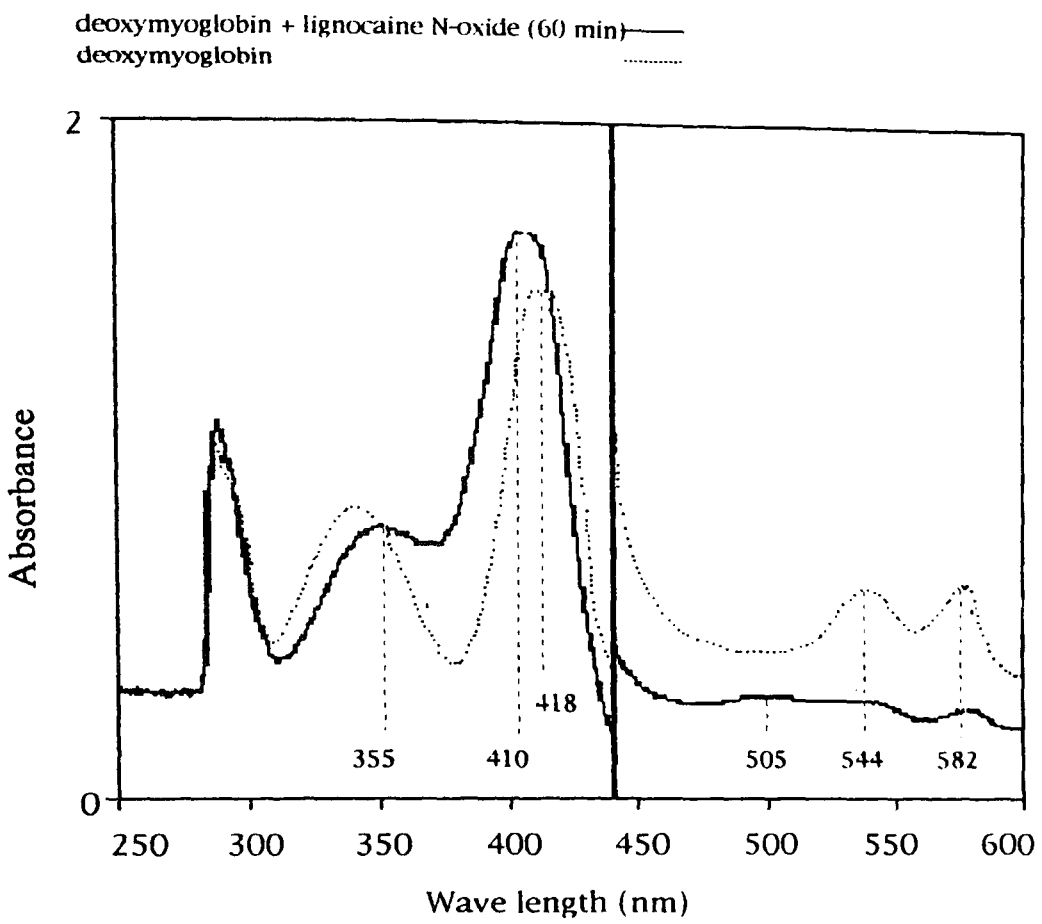


Figure 3-33 UV/visible difference spectrum for deoxymyoglobin alone; and deoxymyoglobin incubated anaerobically with lignocaine *N*-oxide at 37°C after 1 hour. Experimental details were as described in section 2.4.9.

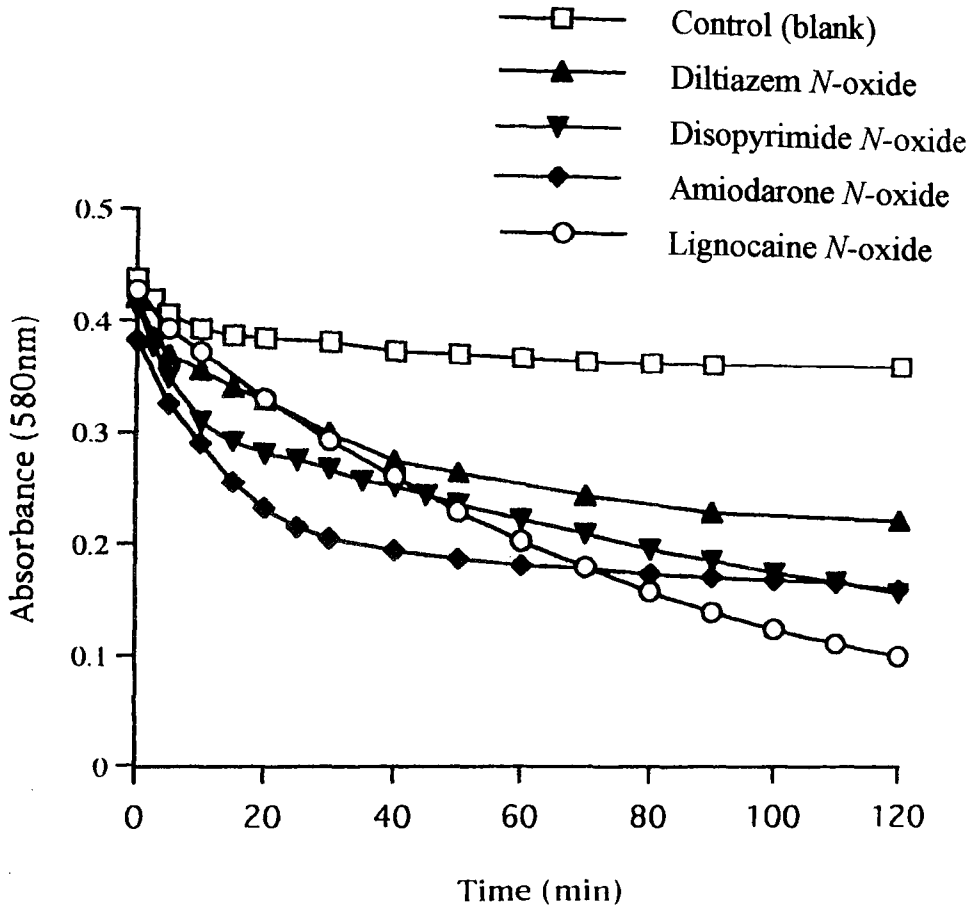


Figure 3-34 Absorbance observed at 580nm when lignocaine *N*-oxide, amiodarone *N*-oxide, diltiazem *N*-oxide or disopyramide *N*-oxide were incubated at 37°C under anaerobic conditions with deoxymyoglobin. Experimental details as described in section 2.4.9.

CHAPTER 4 DISCUSSION 4-2

4.1 HPLC ANALYSIS OF LIGNOCAINE N-OXIDE AND ITS METABOLITES 4-3

4.2 METABOLISM OF LIGNOCAINE *N*-OXIDE 4-4

4.2.1 Enzymology of lignocaine *N*-oxide reduction in liver 4-4

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4.3 MECHANISM OF LIGNOCAINE *N*-OXIDE REDUCTION 4-13

Chapter 4 Discussion

Sudden cardiac death has been a major medical issue for four decades.[196] Cardiovascular arrhythmias are exceedingly complex and the therapeutic utility of most available antiarrhythmic drugs is limited either by lack of consistent efficacy or by the potential for serious side effects. Lignocaine is the drug of choice for the acute treatment of arrhythmias following cardiac arrest.[197] However, the short biological half-life, narrow therapeutic range and a rapid metabolic clearance have limited the use of lignocaine in the controlling of arrhythmias or preventing cardiac events.[198, 199] There is a need for agents with prolonged action and less systemic toxicity, and prodrugs with heart specific activation would appear to fulfil this role.

The possible use of aliphatic tertiary amine *N*-oxides as bioreductive agents in the chemotherapy of hypoxic regions has been extensively studied and reviewed by Patterson (1993).[68] Patterson *et al.* also reported that aliphatic amine *N*-oxides of DNA intercalators with alkylamino sidechains such as bis-alkylamino substituted anthraquinone, anthrapyrazole and anthracene and mono-acridinone possess low aerobic toxicity can be metabolised to their potent cytotoxic parent compounds, the DNA binding and tertiary amines.[67] These *N*-oxides of DNA affinic, cytotoxic agents can be bioreduced selectively in the hypoxic tumour cells are recognised as potential bioreductive drugs.[200]

Ischaemic heart tissue is well recognised as a region of transient hypoxia.[201] The ability of hypoxic cardiac tissue to convert antiarrhythmic *N*-oxide prodrugs into active tertiary amines was proposed and studied using lignocaine *N*-oxide as a model compound. The myocardial, hepatic and systemic metabolism of lignocaine and its mechanism of reduction was studied in detail to assist the evaluation of the suitability of lignocaine *N*-oxide as an antiarrhythmic prodrug.

4.1 HPLC analysis of lignocaine n-oxide and its metabolites

Given that lignocaine *N*-oxide metabolism had not previously been described, a novel HPLC analytical method was developed here to give simultaneous separation, identification and quantification of the highly polar lignocaine *N*-oxide; and the aliphatic amine bases lignocaine and monoethylglycine xylidide (MEGX), an anticipated polar metabolite of lignocaine, in various biological matrices. Aliphatic tertiary amine *N*-oxides are highly polar which would partly account for difficulties in their detection and analysis in biological samples, since they are extracted poorly by many organic solvents, partition poorly into chromatographic stationary phases and can be thermolabile.[178] A rapid, simple acid-acetonitrile deproteination/extraction sample pre-treatment procedure prior to a reversed-phase HPLC system has been applied in the routine study of the fate of lignocaine *N*-oxide in tissues, subcellular fractions, cells, urine and non-biological samples. This method was showed to be stable, sensitive and reproducible and provided good recovery of lignocaine *N*-oxide and its metabolites of interest to this study.

Reversed-phase HPLC which is capable of polar compound analysis has also been used for the analysis of other *N*-oxides including chlorpromazine *N*-oxide, amitriptyline *N*-oxide, and imipramine *N*-oxide in *in vivo* metabolism studies.[113, 116, 184, 202, 203, 204, 205]

Detection of metabolites in lignocaine N-oxide metabolism

All samples analysed in the HPLC analysis as described in Chapter 2 were examined against standards of lignocaine *N*-oxide, glycine xylidide (GX) and MEGX throughout the study. Lignocaine was the only detected metabolite found in lignocaine *N*-oxide metabolism under anaerobic conditions. The metabolism of lignocaine has been extensively studied and was reported to be species dependent.[41, 35, 206, 207, 208] The main reactions described are *N*-deethylation, hydrolysis and ring hydroxylation.[209] Lignocaine is metabolised *via* hydroxylation of the aromatic ring

and the methyl group to give MEGX and 3-hydroxy lignocaine in human hepatic microsomes. In rat hepatic microsomes, lignocaine is sequentially metabolised to MEGX and GX.[209] In this study, no MEGX, GX or 3-OH lignocaine was detected in rat liver (subcellular fractions), rat heart (cellular, tissues, whole heart and perfusates), urine of lignocaine *N*-oxide *i.p.* dosed rats, rabbit myocytes or human liver P450 phenotyped microsomes.

Evidence of the formation of lignocaine N-oxide

The formation of *N*-oxides from aliphatic tertiary amine functional groups has been reported to be a major metabolic pathway for many tertiary amine compounds.[71, 210, 211] Patterson *et al* identified lignocaine *N*-oxide from an incubation of lignocaine with NADPH supplemented rat liver microsomes but as a very minor metabolite.[177] In this report, lignocaine *N*-oxide was extracted from the microsomes, separated by TLC and was identified using chemical ionisation mass spectrometry (CIMS). This is the only report relating to lignocaine metabolism to lignocaine *N*-oxide, an extremely water soluble Phase 1 metabolite.

4.2 Metabolism of lignocaine *N*-oxide

4.2.1 Enzymology of lignocaine *N*-oxide reduction in liver

The metabolism of lignocaine *N*-oxide was first studied in liver since it is a rich source of drug metabolising enzymes including cytochrome P450s.

Lignocaine N-oxide metabolism in rat liver microsomes

The occurrence and mechanism of lignocaine *N*-oxide reduction to lignocaine has not been investigated previously. It was established that lignocaine *N*-oxide can be quantitatively reduced to lignocaine by NADPH supplemented rat liver microsomes under hypoxic and anoxic conditions. No other metabolites (*e.g.* *N*-dealkylation to

MEGX) were detected following incubations of lignocaine under conditions of hypoxia up to one hour (Figure 3-5). This *N*-oxide reduction reaction was NADPH dependent, sensitive to oxygen, and was inhibited by carbon monoxide and cyanide. This suggests a NADPH dependent *N*-oxide reductase, cytochrome P450 mixed function oxidase, may be responsible for lignocaine *N*-oxide metabolism in rat liver microsomes. The requirement for a native enzyme involvement in lignocaine *N*-oxide reduction was demonstrated by the total inhibition of lignocaine formation after heat denaturation of microsomes.

Substrate binding of lignocaine to rat liver microsomes

The interaction between lignocaine *N*-oxide and either oxidised or reduced rat liver microsomal suspensions showed a typical type I spectral changes (417 nm). This suggests lignocaine *N*-oxide may interact with the protein moiety of the cytochrome P450 haemoprotein. There was no evidence (type II difference spectrum) to support the direct coordination of lignocaine *N*-oxide to the reduced haem iron (Fe^{2+}) of cytochrome P450.[154, 159, 160, 161, 162]

Lignocaine, also known to be metabolised by P450 enzymes,[209] produced similar type I difference spectra with the oxidised and reduced rat liver microsomes. As shown in Figure 3-22 and 3-23, the maximum absorbance of all substrate-rat liver microsomes binding spectra were proportional to the substrate concentrations. In rat liver microsomes, lignocaine *N*-oxide seems to have more binding to the reduced microsomes (Ks =1.5 μM) than oxidised microsomes (Ks =250 μM). This result is further supported by the similar Km value calculated from the enzyme kinetics of lignocaine *N*-oxide in the rat liver S9 fractions (250 μM see section 3.2.2).

Lignocaine N-oxide metabolism by phenotyped human liver microsomes

Human liver microsomes contain multiple forms of P450 and genetic polymorphisms are recognised in drug metabolism.[212] A panel of phenotyped human liver microsomes containing different ratios of cytochrome P450 isozymes were used to investigate the correlation between lignocaine *N*-oxide reduction and cytochrome

P450 isozymes. Lignocaine *N*-oxide was showed to be reduced by some of the phenotyped microsomes under anaerobic conditions. Lignocaine *N*-oxide reduction, unlike lignocaine dealkylation known to be catalysed by CYP3A4, showed no correlation to any specific cytochrome P450 isoforms. This result suggested a wide range of isoforms might be responsible for *N*-oxide reduction and the possible occurrence of lignocaine *N*-oxide reduction in humans.[209] Unlike AQ4N, which is reduced by the CYP2C8 and CYP3A family, there may not be a specific active site of cytochrome P450 isoforms for lignocaine *N*-oxide.[213]

Enzymology of lignocaine N-oxide reduction in liver -role of cytochrome P450

According to this study, lignocaine *N*-oxide reduction in rat liver microsomes is enzymic and is mediated by cytochrome P450 in the liver. This is supported by the direct *N*-oxide reduction of lignocaine *N*-oxide with purified cytochrome P450 reductase in the presence of haem.

Previous work relating to several tertiary amine *N*-oxide reduction including tiaramide *N*-oxide, imipramine *N*-oxide and dimethylaniline *N*-oxide has been described by Kato *et al.* [108, 109, 214] They suggested that under anaerobic conditions in the presence of either NADH or NADPH, these *N*-oxides may be metabolised to amines by liver homogenates, microsomes or cytochrome P450. The microsomal NADPH dependent *N*-oxide reductase was induced by phenobarbitone, was oxygen sensitive and inhibited by carbon monoxide, n-octylamine and various P450 inhibitors. The results of the present study support the suggestion that cytochrome P450 is involved in *N*-oxide reduction. Iwasaki *et al.* studied tiaramide *N*-oxide reduction using a reconstituted purified cytochrome system that provided some proof of the ability of cytochrome P450 mediated *N*-oxide reduction and identified the direct binding of *N*-oxide to the cytochrome haem.[110] The present study shows that lignocaine *N*-oxide metabolism is directly correlated to cytochrome P450. Cytochrome b_5 and cytochrome P450 level were both significantly decreased by cobalt treatment which correlated with inhibition of reduction.

The above results established the feasibility of lignocaine *N*-oxide reduction under

hypoxic conditions and indicated the involvement of cytochrome P450 under the requirement for low oxygen concentration.

Lignocaine N-oxide reduction in cobalt treated rat liver microsomes and haem oxygenase

Lignocaine *N*-oxide metabolism was inhibited as the cytochrome P450 level was suppressed in the liver microsomes prepared from the cobalt *i.p.* dosed rats. Haem oxygenase, a microsomal enzyme capable of haem metabolism was concomitantly induced by the cobalt treatment. Haem metabolism is a main pathway for the removal of haem during the turnover of haemoproteins such as haemoglobin, myoglobin and cytochrome P450s and thus will reduce the cellular haem level.

Haem oxygenase is known as one of the heat shock proteins (HSP), a group of intracellular proteins which confer a protective effect during stress and are upregulated by heat and other physiological stress factors including free radicals and inflammatory mediators.[169] The induction of HSP during oxygen stress was reported in myocardial ischaemia/reperfusion,[215] kidney hypoxia and tumour hypoxia with the reduction of cellular haem level. Haem oxygenase that can be induced by a variety of stimulants-Co, Cd, Zn, Sn, haem and hormones, has two isoforms- HO-1 and HO-2.[173] The HO-1 gene that has heat shock regulatory elements, can be induced *in vivo* up to 100 fold during oxidative stress, is responsible for the decrease of total hepatic P450 levels.[165]

Haem oxygenase is not a haemoprotein, instead it will bind to haem in a 1:1 molar ratio as a transitory haemoprotein, and requires microsomal NADPH-cytochrome P450 reductase in the haem oxidation process. Haem degradation products, *i.e.* bile pigments, were reported to be antioxidants and free radical scavengers that can protect tissue damage from oxygen stress.[168] Haem oxygenase has an absolute requirement for NADPH and dioxygen, can serve as a mono-oxygenase and can be inhibited by carbon monoxide. Under anaerobic conditions, lignocaine *N*-oxide can be reduced to lignocaine by cytochrome P450 reductase, haem and NADPH with or without haem oxygenase. This suggests that haem oxygenase is not responsible for lignocaine

N-oxide reduction in the ischaemic/hypoxic heart but may still be involved in an endogenous protective mechanism for oxygen stress in the heart. It is interesting to speculate that lignocaine *N*-oxide could not bind to haem within the HO catalytic site due to the presence of carboxylic acid groups from amino acids flanking the haem pocket. The negative charge on these carboxylic acids may repel N^+-O^- moiety of lignocaine *N*-oxide.

4.2.2 Lignocaine *N*-oxide metabolism in the heart

Lignocaine N-oxide metabolism in the rat heart

The potential of lignocaine *N*-oxide as an antiarrhythmic prodrug was first determined by the metabolism of this agent in hypoxic heart homogenate. Lignocaine *N*-oxide was quantitatively metabolised to lignocaine in rat heart homogenate and S9 fraction, composed of heart cytosol and sarcoplasmic reticulum, under anaerobic conditions with the supplement of NADPH. Lignocaine was the only metabolite found in the lignocaine *N*-oxide reduction study in anaerobic rat hearts. No further lignocaine metabolism was detected in an anaerobic incubation of lignocaine with rat heart homogenate up to 90 minutes (Figure 3-5).

A significant decrease (40%) in lignocaine *N*-oxide reduction after heat denaturation of heart S9 fraction demonstrated the importance of enzyme/proteins in its metabolism. Inhibitors of haemoprotein mediated reduction including oxygen, carbon monoxide and cyanide produced a considerable decrease of lignocaine *N*-oxide reduction in rat heart S9 fractions. The diminution of *N*-oxide reduction but not its complete abolition, suggests the existence of a non-enzymic pathway of lignocaine *N*-oxide reduction. The total inhibition of lignocaine *N*-oxide reduction by carbon monoxide indicates that all pathways to the reduction of the *N*-oxide require a haem centred iron.

Reduction of lignocaine *N*-oxide in heart sarcosomes was not observed. This may

have been due to the low cytochrome P450 content in the sarcosomes although P450 expression in heart has been poorly investigated.[174] The fact that lignocaine *N*-oxide was extensively reduced in the heart cytosolic fraction in the absence of air suggesting that cytosol is an important site for lignocaine *N*-oxide reduction in myocardium.

Lignocaine N-oxide reduction by purified bovine myoglobin

Myoglobin is a globular protein with a molecular weight about 17,200 and 153 amino acids containing one protohaem.[216] It is an intracellular haemoprotein that can reversibly bind oxygen and is found in high concentrations in red skeletal muscle and heart of most vertebrate species. In heart cytosol, myoglobin is a major haemoprotein. In this study myoglobin was shown to be capable of reducing lignocaine *N*-oxide to lignocaine under anaerobic conditions. Specifically a quantitative reduction of lignocaine *N*-oxide to lignocaine by purified bovine deoxymyoglobin under anaerobic incubation was measured by HPLC. The *N*-oxide reduction was highly oxygen sensitive with a total inhibition at oxygen tension of 5%. This was likely to be due to the high binding affinity of myoglobin for oxygen. In the lignocaine *N*-oxide reduction process, myoglobin was considered to act as an *N*-oxide reductase and carried out lignocaine *N*-oxide reduction in the anoxic heart.

Unlike myoglobin, lignocaine *N*-oxide was not reduced by haemoglobin. This may be due to the structural difference between haemoglobin, a tetrameric haemoprotein, and myoglobin, a monomeric haemoprotein, preventing the accessibility of lignocaine *N*-oxide to the haem sites in the tetrameric protein.

No studies relating to myoglobin and aliphatic tertiary amine *N*-oxide reduction under reducing conditions have been reported previously. Gorrod and Patterson have demonstrated that *N,N*-dialkylaniline *N*-oxides were reduced by haemoglobin.[217] The N-oxides of *N,N*-dimethylaniline, *N,N*-dimethylamino-azobenzene, indicine and imipramine were reported to be reduced in whole red blood cells, or by oxyhaemoglobin.[101, 106] It is obvious that due to the conformational difference, myoglobin appears to have a very different reaction mechanism to haemoglobin.

Non-enzymic reduction of lignocaine N-oxide

The non-enzymic catalysis of lignocaine *N*-oxide to lignocaine in heart cytosol was further investigated using haemin, a basic haemoporphyrin, in the presence of inorganic iron. Lignocaine *N*-oxide was metabolised by haemin in the presence of both ascorbic acid and NADPH. Non-enzymic *N*-oxide reduction of indicine *N*-oxide and trimethylamine *N*-oxide by inorganic Fe^{2+} has been reported.[218] However, lignocaine *N*-oxide reduction was not facilitated by Fe^{2+} or Fe^{3+} in the presence of ascorbic acid and NADPH. It is evident that lignocaine *N*-oxide can be metabolised through a non-enzymic pathway that contributes to more than half of lignocaine *N*-oxide conversion in the anaerobic rat heart cytosol. The possible mechanism of this is discussed in the next section.

Lignocaine N-oxide reduction in the isolated rat heart

Lignocaine *N*-oxide was found to be reduced to lignocaine in the isolated rat hearts under hypoxic conditions. This is the first time that evidence has been obtained relating to the myocardial reduction of lignocaine *N*-oxide in the intact heart.

A significant amount (46%) of lignocaine *N*-oxide was reduced in the hypoxic/anoxic isolated rat heart. A steady (normal) cardiac rhythm was maintained in the undosed heart. Similarly, hearts perfused with lignocaine or lignocaine *N*-oxide maintained normal cardiac rhythms. A change from oxygenated, hypoxic to anoxic condition was produced by interrupting the perfusion flow. After cessation of the oxygenated perfusion medium, normal and lignocaine perfused isolated hearts stopped in 1-2 minutes, whilst lignocaine *N*-oxide perfused isolated hearts had a prolongation of the heart beat for up to 8 minutes. Under normal (oxic) perfusion, no lignocaine was found in the perfusion fluid from lignocaine *N*-oxide perfused heart; no lignocaine *N*-oxide was found in the perfusion fluid from the lignocaine perfused heart. Lignocaine derived from lignocaine *N*-oxide was found only in the anoxic heart. This suggests lignocaine *N*-oxide was metabolised selectively in the hypoxic/anoxic heart.

An *in vivo* study on lignocaine *N*-oxide was previously carried out in sheep by McIntosh and Kane in Strathclyde Institute for Drug Research (1991).[176] The cardiac electrophysiological effects recorded in sheep hearts demonstrated that lignocaine *N*-oxide was not active under normal conditions, and showed that it was no less potent than lignocaine under simulated ischaemic conditions (pO_2 25-30 mmHg).

The *in vitro* studies on lignocaine *N*-oxide perfused through controlled, isolated animal hearts suggested that lignocaine *N*-oxide has the potential of acting as a site-specific antiarrhythmic prodrug that may be activated only in the ischaemic tissues/organs.

Xanthine oxidase

It is known that reperfusion of oxygen to ischaemic cardiac muscle can generate superoxide radicals and damage the heart.[219] This injury is thought to be due to cytotoxic oxygen free radicals generated by xanthine oxidase.[18] Administration of allopurinol, a xanthine oxidase inhibitor, has been observed to diminish the amount of reactive oxygen damage to the heart.[13] Lignocaine *N*-oxide was reduced by purified xanthine oxidase under anaerobic conditions. This was investigated since xanthine oxidase was previously showed to reduce other *N*-oxides.[80] Lignocaine, already a potent antiarrhythmic agent, is reported to be a hydroxy radical scavenger[220] and this may contribute to the protective effects seen by this agent. Xanthine oxidase is a heart cytosolic enzyme so is likely to contribute to the total lignocaine *N*-oxide reduction observed in heart tissue.

4.2.3 *In vivo* metabolism of lignocaine *N*-oxide

Lignocaine *N*-oxide metabolism *in vivo* following its *i.p.* administration to a healthy rat showed less than 5% of the reduction product in the urine. The majority of *N*-oxide was excreted unchanged. This suggests that lignocaine *N*-oxide may be stable to metabolic reduction under oxic condition *in vivo*.

The *in vivo* metabolism of *N*-oxides has not been extensively studied. It has been recognised that compounds containing an aliphatic tertiary amine group may be oxidised to *N*-oxide metabolites; and these *N*-oxides can be reduced to their corresponding amines.[70, 71, 117] Early studies on trimethylamine *N*-oxide suggested that *N*-oxide reduction of this compound is not a major route of metabolism.[84] However, the *in vivo* metabolism of chlorpromazine *N*-oxide, amitriptyline *N*-oxide, indicine *N*-oxide and imipramine *N*-oxide demonstrated that tertiary amine *N*-oxides can be metabolised through *N*-dealkylation and hydroxylation as well as *N*-oxide reduction.[113, 114, 115, 116]

Ideally as a site/diseased state activated prodrug, lignocaine *N*-oxide is expected to be reduced to lignocaine in hypoxic tissues/organs and remain unchanged in normal oxygenated tissues. In this study, lignocaine was not oxidised to lignocaine *N*-oxide in rat liver microsomes or heart homogenate under anaerobic condition. The systemic metabolism of lignocaine *N*-oxide is unknown. Further pharmacokinetic and pharmacodymic studies under controlled or systemic ischaemic conditions might provide detailed information on the bioactivation/inactivation of lignocaine *N*-oxide *in vivo*.

4.3 Mechanism of lignocaine *N*-oxide reduction

Haemoprotein mediated lignocaine N-oxide reduction

Previous sections have demonstrated that haemoproteins, including cytochrome P450 and myoglobin, are capable of lignocaine *N*-oxide reduction under anoxic conditions. The absolute requirement for the absence of oxygen and the requirement of NADPH highlights the importance of the reduced status of haem component. The inhibition of *N*-oxide reduction by oxygen, carbon monoxide and cyanide, as well as a direct *N*-oxide reduction by the reduced haemin further supports a role for the haem binding of lignocaine *N*-oxide as a prerequisite for its reduction.

Mechanism of Lignocaine N-oxide reduction by P450 and other NADPH requiring haem based systems

In this study cytochrome P450 and NADPH have been shown to mediate *N*-oxide reduction with lignocaine *N*-oxide behaving as an alternative oxygen source to molecular oxygen. The *N*-oxide reduction by myoglobin appears slightly different because no external reducing system is required.

We postulate an oxygen atom transfer process between the *N*-oxide and haem centre. Figure 4-1 illustrates a proposed lignocaine *N*-oxide reduction by the haem centre of haemoproteins. First, a one electron reduction of the haemoporphyrin, [Fe(III) to Fe(II)] can be generated by NADPH *via* NADPH cytochrome P450 reductase. (i) The oxygen atom of lignocaine *N*-oxide is coordinated to the Fe(II) atom of the reduced haemoprotein. (ii) At this stage, the hydrophobic region of lignocaine *N*-oxide may also bind to the substrate binding domain of the protein and forms a haem[Fe(II)]-*N*-oxide-protein complex. (iii) With the cleavage of the N-O bond, lignocaine (tertiary amine) and an oxy-ferryl haemoprotein, [oxy-Fe(IV) pair], complex are formed. This ferryl intermediate can be represented as [Fe(II)-O], Fe^{4+}-O^{2-} or [Fe(IV)=O]. In this case, the haem-[Fe(IV)]=O to give a net ionic charge of +2. (iv) Following the transfer of the second electron to the oxy-ferryl haemoprotein complex and the release of one molecule of water, the oxidised haemoprotein [Fe(III)] is formed.

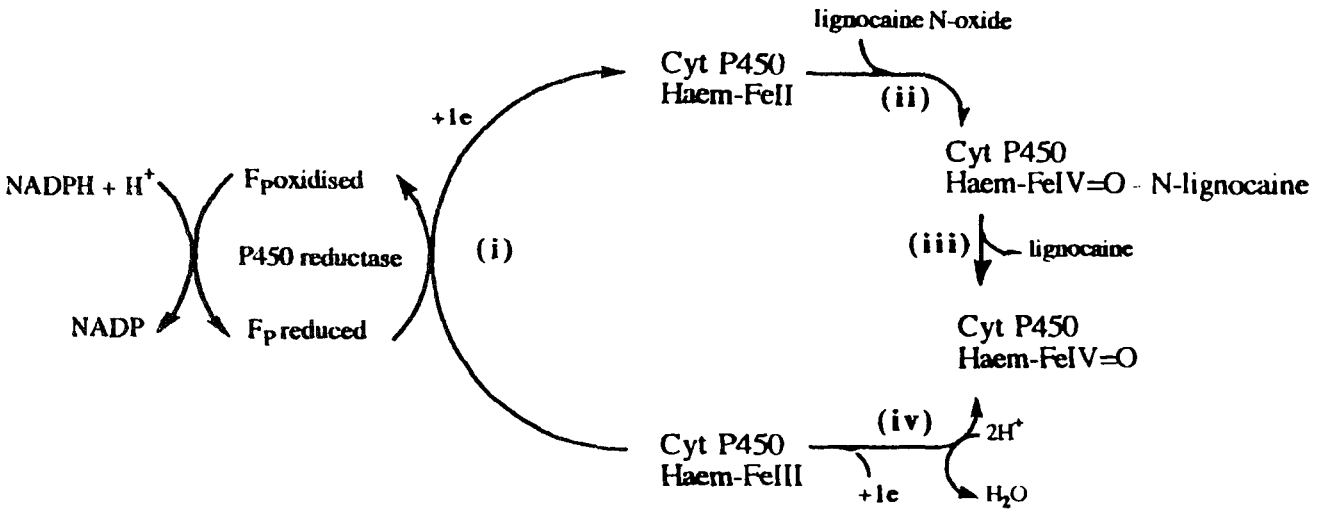

Figure 4-1 Proposed lignocaine *N*-oxide reduction mechanism by haemoprotein:

i. One electron reduction of haem Fe(III) to Fe(II).

ii. The formation of haem [Fe(IV)=O]···N-lignocaine complex.

iii. N-O bond cleavage to form a oxy-ferryl [Fe(IV)=O] haemoprotein intermediate and lignocaine.

iv. Following a second electron transfer, the oxidised haemoprotein [Fe(III], lignocaine and water are formed.

Previously, a mechanism for cytochrome P450 mediated tertiary amine *N*-oxide reduction was proposed by Kato *et al.*[221] The reaction mechanism was described to be NADPH-dependent. Cytochrome P450 is first reduced by NADPH. The *N*-oxide is then coordinated with the reduced cytochrome haem region. After a further one electron reduction of the reduced cytochrome P450-*N*-oxide complex concomitant with a two electron reduction of the tertiary amine *N*-oxide, tertiary amine and water are formed as the final products. In biological systems, the electron donors in the cytochrome P450 mediated *N*-oxide reduction can be cytochrome b_5, NADPH-cytochrome P450 reductase, or other flavoproteins.

Lignocaine N-oxide reduction via myoglobin

Lignocaine *N*-oxide was reduced by deoxymyoglobin to lignocaine under anaerobic conditions as shown by HPLC analysis (see section 3.4.1). During this process, a spectral change of deoxymyoglobin [Fe(II)] to metmyoglobin [Fe(III)] was observed indicating that myoglobin had undergone a one electron oxidation. In anaerobic conditions, with no molecular oxygen to bind to the haem centre of myoglobin, the single oxygen from the *N*-oxide reduction of lignocaine *N*-oxide will be an alternative source of available oxygen. It was rationalised that a ferryl myoglobin, [Fe(IV)=O or oxy-ferryl], intermediate should result from lignocaine-*N*-oxide mediated oxidation of myoglobin *via* a lignocaine-*N*-oxide dependent oxygen atom transfer process (see figure 4-1).

Lignocaine *N*-oxide reduction concomitant with myoglobin oxidation was studied using visible spectrometry. However in the visible spectrum, the oxidation of deoxymyoglobin to metmyoglobin was rapid. Hence measurement of the formation of myoglobin oxidation intermediates, including ferryl myoglobin was not possible. In order to study this oxidation process further, electron spin resonance spectroscopy (ESR) in combination with 5,5-dimethylpyrroline 1-*N*-oxide (DMPO), a spin trapping agent for short lived free radicals[222] was employed.

A complex mixture of ESR signals comprising DMPOX (5,5-dimethylpyrrolidone-2-

oxy-1) free radical, the oxidation product of DMPO,[195] and DMPO/ R$^{\bullet}$, a carbon centred radical was recorded under anaerobic conditions from deoxymyoglobin and lignocaine *N*-oxide incubated in the presence of DMPO (see Figure 3-28 B). As shown in Figure 3-28, this ESR spectrum was not produced by metmyoglobin or lignocaine under aerobic or anaerobic conditions, and deoxymyoglobin and lignocaine *N*-oxide under aerobic conditions. These results showed that the free radical species produced by lignocaine *N*-oxide and deoxymyoglobin under anaerobic conditions did not come from the metmyoglobin, oxidised myoglobin [Fe(III)] or deoxymyoglobin, reduced myoglobin [Fe(II)] alone. However, the presence of both a DMPO/ R$^{\bullet}$ and DMPOX adduct is highly suggestive of the presence of a myoglobin [Fe(IV)=O] species. Such an intermediate would react with DMPO to form both DMPOX and DMPO/ R$^{\bullet}$ products. A similar ESR spectrum containing DMPOX and DMPO/ R$^{\bullet}$ was recorded from DMPO, methylamine and deoxymyoglobin under anaerobic conditions (see Figure 3-28 H). This result suggests that the formation of carbon centred radicals (DMPO/ R$^{\bullet}$) may be a result of the interaction between any alkylamine including lignocaine *N*-oxide and the reduced haem of deoxymyoglobin.

Using the ESR spectroscopy and visible spectroscopy results obtained, the proposed mechanism of myoglobin mediated lignocaine *N*-oxide reduction can be summarised as follows:

1. In the absence of oxygen, deoxymyoglobin [Fe(II)=O] binds to lignocaine *N*-oxide to form a lignocaine-oxy-ferryl myoglobin [Fe(IV)=O$^{\cdot\cdot}$ lignocaine] intermediate. The presence of reactive oxygen species and alkyl radical species (trapped by DMPO as DMPO/OH and DMPO/R respectively) indicates that when lignocaine-*N*-oxide reacts with deoxymyoglobin a highly reactive myoglobin intermediate, such as ferryl myoglobin, is formed. DMPO can also be oxidised to DMPOX due to the peroxide nature of the ferryl intermediate.

   Deoxymyoglobin [Fe(II)] + lignocaine *N*-oxide →

   lignocaine-oxy-ferryl myoglobin [Fe(IV)=O$^{\cdot\cdot}$ lignocaine]

2. The formation of metmyoglobin by lignocaine-*N*-oxide (as measured by visible spectroscopy (see figure 3-34) and the concomitant quantitative reduction of lignocaine-*N*-oxide to lignocaine (as shown by HPLC analysis, see figure 3-25) indicates that the ferryl myoglobin intermediate predominantly decomposes by loss of a hydroxyl radical-like species. This reactive oxygen species appears to predominantly decompose to water or by oxidative modification *e.g.* hydroxylation of myoglobin apoprotein. Such myoglobin oxidation products would not be detected by the spectroscopic methods used in this study.

Lignocaine-oxy-ferryl myoglobin [Fe(IV)=O$^{\cdot\cdot}$lignocaine]→

lignocaine + metmyoglobin [Fe(III)] + H_2O

Enzyme kinetic constants of lignocaine N-oxide reduction in rat liver microsomes and heart S9 fractions

The Km and Vmax of lignocaine *N*-oxide metabolism was determined in rat liver microsomes and rat heart S9 fractions. Similar results were obtained, with Km of 250.0 and 269.8 μM; and Vmax of 0.99 and 1.11 nmole/min/mg in the liver and heart, respectively. According to previous results, the mechanisms of lignocaine *N*-oxide reduction in rat liver microsomes and heart S9 fractions are both complex and different in the enzymes and factors involved. This similarity of the Km and Vmax can only represent similarities between lignocaine *N*-oxide reduction in liver microsomes and heart S9. It is remarkable that the kinetic attributes in the tissues are so similar and suggests that all the processes involved share a common rate limiting step. Thus may be as a result of the general requirement for haem binding of lignocaine *N*-oxide.

Tertiary aliphatic amine as antiarrhythmic prodrugs

The interaction of myoglobin with other tertiary amine *N*-oxides of antiarrhythmic compounds were also studied under anoxic conditions. Similar visible spectral

changes to those with lignocaine *N*-oxide were observed for the anaerobic incubation of amiodarone *N*-oxide, diltiazem *N*-oxide and disopyramide *N*-oxide with deoxymyoglobin. It is likely that all these *N*-oxides undergo a similar mechanism of myoglobin mediated reduction as illustrated with lignocaine *N*-oxide.

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Chapter 5 Conclusions

This study is based on a proposal concerning the feasibility of using aliphatic tertiary amine *N*-oxides as antiarrhythmic agent prodrugs. Lignocaine was selected as a candidate for the prodrug development because it is the drug of choice for ventricular arrhythmia, a condition associated with ischaemic episodes leading to regions of transiently hypoxic heart tissue. The present effort has been focused on the use of lignocaine *N*-oxide as a hypoxia-mediated bioreducible antiarrhythmic durg. Lignocaine *N*-oxide, a non-active and polar derivative of lignocaine, has been demonstrated to be activated (bioreducted) selectively in the ischaemic/hypoxic heart to its parent compound, lignocaine.

A previous *in vivo* study of lignocaine *N*-oxide by McIntosh and Kane in Strathclyde Institute for Drug Research (1991)[176] demonstrated that lignocaine *N*-oxide was biologically inert under normal conditions, but was active during ischaemia. This preliminary result suggested that lignocaine *N*-oxide might have use as a potential antiarrhythmic prodrug.

In this study, the oxygen sensitivity was studied to justify whether the bioactivation of lignocaine *N*-oxide can be regulated by the prevailing oxygen tension in the target tissue. In a arrhythmic heart, the prodrug activation would be triggered by the ischaemic state of the heart and terminated as the oxygen content in the heart returned to a more normal level. This would therefore be expected to achieve a controlled release and site-specific active drug delivery.

An HPLC method was developed to study the metabolism of lignocaine *N*-oxide. The simple and rapid method of analysis of lignocaine and its metabolites was demonstrated to have good reproducibility, stability and recovery.

5. 1 A Summary of the metabolism of lignocaine *N*-oxide

It was found in this study that lignocaine *N*-oxide can be reduced to its active parent compound, lignocaine, in the absence of oxygen. No other metabolites were detected under anaerobic conditions. Under anaerobic conditions, no metabolism of lignocaine was demonstrated in rat liver microsomes and heart S9 fractions. The *in vitro* lignocaine *N*-oxide reduction was found to occur under anaerobic conditions in NADPH supplemented rat liver homogenates, microsomal suspensions; rat heart homogenates, cytosolic solutions; human phenotyped cytochrome P450 isoforms; purified enzymes- cytochrome P450 reductase and xanthine oxidase.

Lignocaine *N*-oxide was anaerobically metabolised by the NADPH/ascorbate reduced protohaem (haemin); and deoxymyoglobin.

In hypoxic/anoxic isolated rat hearts, lignocaine *N*-oxide was found to be reduced to lignocaine.

No lignocaine *N*-oxide reduction was found in the oxidised/reduced inorganic iron, NADPH/ haem supplemented haem oxygenase and reduced haemoglobin systems under anaerobic conditions.

5.1. 1 Enzymology of lignocaine *N*-oxide reduction

The reduction of lignocaine *N*-oxide has been shown to be both enzymic and non-enzymic. It was found that the lignocaine *N*-oxide reduction is NADPH dependent, oxygen sensitive and can be suppressed by CO, CN^- and protein denaturation (heat). It can be inhibited through the suppression of P450 and b_5 levels in rat liver microsomes; mediated by haemoproteins (*i.e.* cytochrome P450 and myoglobin), non-

haemoprotein enzymes (*i.e.* cytochrome P450 reductase and xanthine oxidase), or NADPH/ascorbic acid reduced protohaem (haemin).

According to the postulated P450 mediated *N*-oxide reduction mechanisms suggested in Figure 4-1 and the ESR study of myoglobin in section 4.3, the mechanisms of lignocaine *N*-oxide metabolism *via* haemoprotein can be summarised as follows: (i) The oxygen atom of lignocaine *N*-oxide is coordinated to the Fe(II) atom of the reduced haemoprotein. (ii) A haem[Fe(II)]-lignocaine *N*-oxide-protein complex is formed. (iii) Following the cleavage of the N-O bonding, lignocaine and an intermediate of an oxy-ferryl haemoprotein [oxy-Fe(IV) pair] complex is formed. (iv) With the release of one molecule of water, the oxidised haemoprotein [Fe(III)] is formed. In the P450 system, the 2 electron involved in the reduction of the haemoprotein in step (i) and (iv) can be provided by cytochrome b_5, NADPH-cytochrome P450 reductase (a microsomal flavinprotein), or other proteins. The reduction of myoglobin does not require on external electron donor due to the rapid oxidation of the deoxymyoglobin.

5.1. 2 Phenotyped cytochrome P450 isoforms

Lignocaine *N*-oxide can be metabolised by several phenotyped human liver microsomes. However, there was no direct correlation between lignocaine *N*-oxidation and a panel of 12 human liver phenotyped cytochrome P450's. The major metabolism of lignocaine was oxidation (*N*-dealkylation) in human and was identified to be mediated by *CYP3A4*.[209] This suggested that lignocaine *N*-oxide reduction may not be isoform specific but that lignocaine *N*-oxide fits the active sites of most cytochrome P450's.

5.1. 3 Other enzymes

Three purified enzymes, NADPH-cytochrome P450 reductase, a flavin protein, xanthine oxidase and haem oxygenase, were examined to determine their potential of being an *N*-oxide reductase.

Flavin proteins have been reported to be involved in the electron transfer process in *N*-oxide reduction mechanisms.[128] NADPH-cytochrome P450 reductase was demonstrated to have direct interaction with lignocaine *N*-oxide with a supplement of NADPH and/or haem to form lignocaine.

Xanthine oxidase,[18] which plays an important role in superoxide radical formation and myocardial ischaemia/reperfusion injury, was capable of reducing lignocaine *N*-oxide to lignocaine under anaerobic conditions. It may be partially responsible for lignocaine *N*-oxide metabolism in the ischaemic /hypoxic heart. Whether lignocaine *N*-oxide can interact with xanthine oxidase to generate superoxide radicals during reperfusion was not studied and will require further investigation.

Haem oxygenase associated with NADPH-cytochrome P450 reductase can react with molecular oxygen and mediates the microsomal haem degradation to bile pigments.[169] Haem oxygenase (isoform HO-1), reported to act as mono oxygenase, failed to reduce lignocaine *N*-oxide under anaerobic conditions.

5. 2 Lignocaine *N*-oxide as an antiarrhythmic prodrug

In this study, lignocaine *N*-oxide was found to be a potential antiarrhythmic prodrug. A list of findings that support the use of lignocaine *N*-oxide as an antiarrhythmic prodrugs are as follows:

1. The inert nature of lignocaine *N*-oxide as a prodrug (*e.g.* decreased dose-related

problems, such as adverse side effect and narrow therapeutical index).

2. High recovery of lignocaine *N*-oxide in the urine of normal rats, indicating low metabolism of the prodrug in normal (oxic) tissues. This was based on the findings that: (i) no secondary metabolites of either lignocaine or lignocaine *N*-oxide were found; (ii) *N*-Oxidation is not a major metabolism route of lignocaine.
3. Hypoxia mediated activation of lignocaine *N*-oxide suggesting a (disease) state-specific controlled release of the prodrug in the hypoxic heart. Lignocaine *N*-oxide reduction was oxygen sensitive. A self-regulating mechanism of the prodrug release triggered by the oxygenated condition of the tissue/organ (heart) is proposed.

An important but unexplained effect relating to lignocaine *N*-oxide reduction in the isolated hypoxic/anoxic rat hearts that extended the heart beating time after stopping the oxygen supply for up to 8 minutes, was found and requires further investigation.

Future work

In this study, I have demonstrated that lignocaine as well as other tertiary amine antiarrhythmic agents modified as *N*-oxides may be reduced to their active parent compounds under hypoxic conditions. Further experiments on lignocaine *N*-oxide in ischaemic heart animal models will provide more information concerning the activity of these agents *in vivo*.

Previously, the prodrug approach of using aliphatic tertiary amine *N*-oxide as bioreductive anti-cancer agents has been extensively studied.[67, 68] The application of using aliphatic tertiary amine *N*-oxides as bioreductive prodrugs in ischaemia in CNS or other therapeutic fields have not been studied.

The fate of orally dosed tertiary amine *N*-oxide (*e.g.* nicotine-1'-*N*-oxide and chlorpromazine *N*-oxide) have been studied.[113, 107] The metabolism and excretion of

lignocaine *N*-oxide though different administration routes (*e.g.* oral, intravenous or intramuscular) may require further investigation.

The prodrug approach using lignocaine *N*-oxide or other *N*-oxide prodrugs in the hypoxic region besides tumour and heart can be considered. Ischaemia in chronic and acute wounds may be another region where a controlled-released drug/prodrug delivery system could provide adequate site-specific treatment.

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APPENDIX 2

APPENDIX I PROTEIN ASSAY.....2

APPENDIX II CYTOCHROME P450 AND b_5 MEASUREMENT 4

REFERENCES: 5

Appendix

Appendix I Protein assay

The protein concentration of rat liver and heart subcellular fractions was determined using the Bio-Rad protein assay which is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs (Riesner *et al*, 1975[1]; Sedmak *et al.*, 1977[2; 3]). Bovine serum albumin (BSA) was used as the protein calibration standard. The dye reagent, Coomassie blue, was diluted (1 : 5) with distilled water and filtered through Whatman filter paper (No.1) at room temperature (20-25°C). Protein standards (0.1-0.8 mg/ml BSA) were prepared in clean dry test tubes. The colour of the BSA standards was developed by addition of the dye reagent (5 ml) to aliquots of the BSA standards (0.1 ml) and appropriately diluted subcellular fractions (1 : 100 for liver microsomes). The tubes were vortex mixed and allowed standing for 10 minutes at room temperature. The absorbance was measured (595 nm) against a dye blank solution. A plot of the absorbance versus the concentration of the BSA standards enabled the concentration of the subcellular fraction to be determined. A typical BSA calibration curve is shown in Fig. A-1.

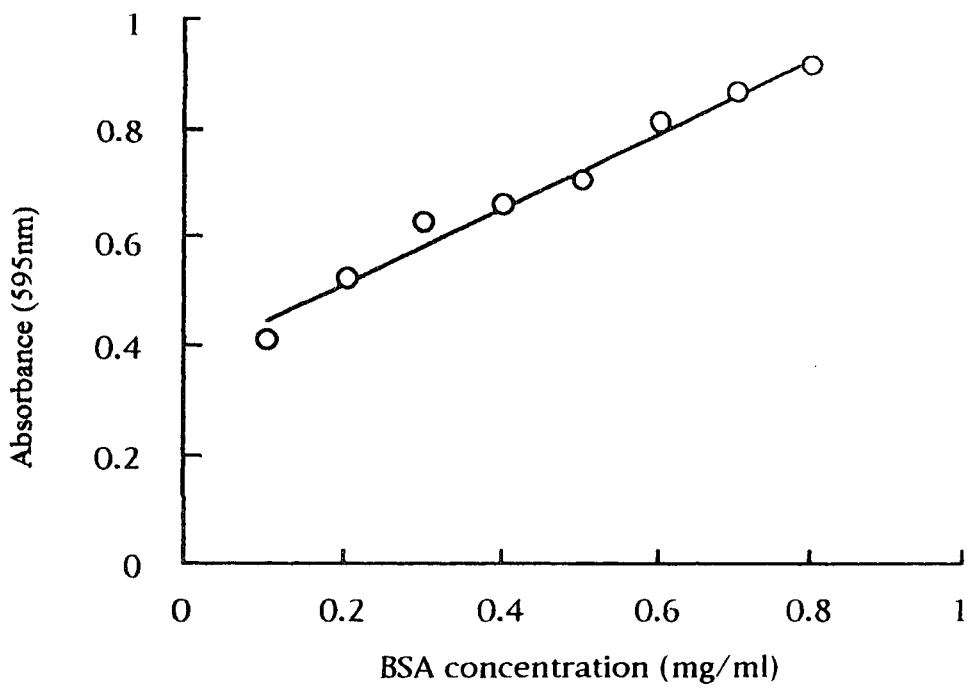

Figure A-1 Bovine serum albumin (BSA) protein standard graph.
Experimental details as described in Appendix 1.
Linear equation: Y=0.3749+0.7182X
Correlation coefficient: r=0.9909

Appendix II Cytochrome P450 and b_5 measurement

(a) Cytochrome b_5

Ferric cytochrome b_5 has a maximum absorbance at 413 nm with an extinction coefficient of 117 mM^{-1} cm^{-1}, while the haemoprotein is characterised by three distinct maxima at 424, 526 and 555 nm with extinction coefficients of 171, 13.4 and 25.6 mM^{-1} cm^{-1}, respectively[4, 5]. Cytochrome b_5 reduction is routinely monitored using wavelength pairs of 414-424 nm (185 mM^{-1} cm^{-1}), 424-500 nm (130 mM^{-1} cm^{-1}) and 557-567 nm (19 mM^{-1} cm^{-1}).

The washed microsomal preparation (1.0 ml) was diluted with phosphate buffer (pH 7.4, 4.0 ml) to give a suspension containing 0.1 g of original liver in 1.0 ml. The suspension was divided between stoppered glass cuvettes as standard and samples. A base line was recorded between 390 and 600 nm. The contents of the sample cuvette were treated with sodium dithionite (about 5 mg). The difference spectrum between the reduced and the oxidised cytochrome b_5 was recorded between 390 and 600 nm. The difference between the absorbance at 424 nm and that at 470 nm was measured and the concentration expressed as $\triangle$424 nm-470 nm/0.5 g of original tissue.

(b) Cytochrome P450

The contents of both cuvettes from the cytochrome b_5 determination were reduced by the addition of sodium dithionite (about 5 mg). The sample cuvette was treated with a stream of carbon monoxide for about 30 seconds. The stoppered cuvettes were inverted twice and the difference spectrum between the reduced cytochrome P450 and the reduced carbon monoxide complexed material was recorded between 390 and 600 nm. The difference between the absorbance at 450 nm and that at 490 nm was measured and the final value recorded as $\triangle$450 nm – 490 nm/0.5g of original tissue.

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